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LABORATORY METHODS
OF
BIOCHEMISTRY

LABORATORY METHODS
OF
BIOCHEMISTRY

BY

A. BERTHO AND W. GRASSMANN

TRANSLATED BY

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WITH 33 FIGURES IN THE TEXT
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PREFACE

The student of modern biochemical research has been obliged, until the present time, to acquire his knowledge of the subject, its aims, domain, methods and results from original publications in the journals, from hand-books and from the text-books of theoretical and practical physiology in which biochemical information is widely scattered. In publishing this book on practical biochemistry, following the procedure adopted in Gattermann and Wieland's *Laboratory Methods of Organic Chemistry*, the authors feel assured that they are fulfilling the urgent wishes of many who desire to make a close study of biochemistry. At the same time they wish to encourage, in those universities and colleges where biochemistry has often been neglected or only incompletely studied, a more worthy treatment of the subject.

In co-operation with E. Waldschmidt-Leitz, H. Kraut and E. Bamann (who also preceded them in this work) the authors have, for years, very successfully used a great part of the material given in this book during a course of practical biochemistry occupying one half day per week in the University of Munich. The advanced students of chemistry who already have a sufficiently complete knowledge of organic chemistry take this course together with young medical students who are more concerned with physiological problems. For these groups of students the course has proved both informative and stimulating. In view of the very great extent of the domain of biochemistry it was necessary to limit severely the choice of practical and theoretical material to be included in the book. The theoretical sections, of course, deal mainly with well established and to some extent settled points only.

The book is divided as follows. First comes a " General Introduction " written by the two authors jointly. The object of this section is to give a general review of those chemical processes which occur in nature and are the subject matter of biochemical investigation. As in Gattermann and Wieland's book a chapter on " General Technique ", in which a detailed description of the use of apparatus and of general experimental procedure is given, precedes the three main divisions of the book. (This chapter is contributed by Grassmann.) The general title for the three main divisions is " Biological Materials and Transformations ". In the chapter on " Substrates " (Grassmann), substances which are studied by organic chemists and the detection and determination of these substances are considered only so far as is necessary for an understanding of biochemical enzymic transformations. The authors did not hesitate to include in this section a few experiments and remarks on the vitamins and hormones, since those chemists for whose use the book is chiefly intended are interested in the constitutions of these very remarkable substances although the study of these constitutions is part of the subject matter of pure organic chemistry. The two remaining sections of the book give an account of what has so far been discovered concerning " Enzymic Hydrolyses " (Grassmann) and " Respiration and Fermentation " (Bertho). It seemed appropriate to divide the sections thus because, as is well known, biochemical investigation is a development of purely enzyme chemistry and because, as is pointed out in the " General Introduction ", enzymic transformations are naturally classified as hydrolytic on the one hand and desmolytic on the other. In these two sections the authors have endeavoured to present to the student as systematically as possible the whole of the material required. The fulfilment of this aim was facilitated by the fact that the authors specialise in these two branches of the subject. The reliability of the great majority of the experimental methods described has been repeatedly checked.

In arranging the practical course it has been found that a successful method is to permit the students to work in small groups under the direction of an Assistant or else under that of a candidate for the doctorate who is familiar with the methods, each experiment occupying a whole day or a half day. Such guidance and supervision are essential for the correct carrying out of the few experiments on animals (insulin, determination of the sugar content of rabbit's blood) included in the course.

A modern laboratory equipped for instruction and research will not lack the apparatus necessary for the course (thermostat, one or two centrifuges, apparatus for measurement of p_H, Van Slyke apparatus, polarimeter, Warburg apparatus) any more than it will lack a combustion apparatus or a Zeisel apparatus.

One of the authors (Bertho) was responsible for the suggestion that this book should be published. Publication was delayed because of adverse circumstances.

ALFRED BERTHO
WOLFGANG GRASSMANN

MUNICH AND DRESDEN, *July* 1935

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ABBREVIATIONS

The abbreviations of the titles of journals are (with a few exceptions) those employed in *British Chemical and Physiological Abstracts.* In the references, the abbreviated title is followed by the year, volume number (in heavy type) and page.

Acta Phytochim. = Acta Phytochimica.
Amer. J. Physiol. = American Journal of Physiology.
Angew. Chem. = Angewandte Chemie (changed from *Z. angew. Chem.*, in 1932).
Annalen = Justus Liebig's Annalen der Chemie.
Ann. Rev. Biochem. = Annual Review of Biochemistry.
Arch. Anat. Physiol. = Archiv für Anatomie und Physiologie.
Arch. exp. Path. Pharm. = Archiv fur experimentelle Pathologie und Pharmakologie.
Beitr. chem. Physiol. Pathol. = Beiträge zur chemischen Physiologie und Pathologie.
Ber. = Berichte der deutschen chemischen Gesellschaft.
Biochem. J. = Biochemical Journal.
Biochem. Z. = Biochemische Zeitschrift.
Chem. Fabr. = Die chemische Fabrik.
Chem.-Ztg. = Chemiker-Zeitung.
Compt. rend. = Comptes rendus hebdomadaires des Séances de l'Académie des Sciences.
Ergebn. Enzymforsch. = Ergebnisse der Enzymforschung.
Ergebn. Physiol. = Ergebnisse der Physiologie.
Ergebn. Vitamin-Hormonforsch. = Ergebnisse der Vitamin- und Hormonforschung.
Helv. Chim. Acta = Helvetica Chimica Acta.
J. Amer. Chem. Soc. = Journal of the American Chemical Society.
J. Bact. = Journal of Bacteriology.
J. Biol. Chem. = Journal of Biological Chemistry.

J. Chim. phys. = Journal de Chimie physique.
J.C.S. = Journal of the Chemical Society.
J. Pharm. Exp. Ther. = Journal of Pharmacology and Experimenta Therapeutics.
J. Physical Chem. = Journal of Physical Chemistry.
J. pr. Chem. = Journal für praktische Chemie.
J.S.C.I. = Journal of the Society of Chemical Industry.
Klin. Woch. = Klinische Wochenschrift.
Kolloid-Z. = Kolloid-Zeitschrift.
Lancet = The Lancet.
Monatsh. = Monatshefte für Chemie und verwandte Teile anderer Wissenschaften.
Nature = Nature.
Naturwiss. = Die Naturwissenschaften.
Nutr. Abs. = Nutrition Abstracts and Reviews.
Proc. Roy. Soc. = Proceedings of the Royal Society.
Science = Science.
Skand. Arch. Physiol. = Skandinavisches Archiv fur Physiologie.
Svensk Kem. Tidskr. = Svensk Kemisk Tidskrift.
Z. Biol. = Zeitschrift für Biologie.
Z. physikal. Chem. = Zeitschrift für physikalische Chemie.
Z. physiol. Chem. = Hoppe-Seyler's Zeitschrift fur physiologische Chemie.
Z. Ver. deut. Zuckerind. = Zeitschrift des Vereins der deutschen Zucker-Industrie.

GENERAL INTRODUCTION

EVERY living organism, whether animal or vegetable, maintains its life by consuming food and converting it into the substances of which its cells are composed.

These *synthetic processes* are, generally, endothermic, *i.e.* they require the application of energy. For the most important synthetic process occurring in the vegetable kingdom, namely, the *assimilation of carbon*,[1] that is to say, the conversion of CO_2 and H_2O into carbohydrate, the energy is supplied directly by the sunlight, the energy of the irradiation being absorbed by the pigment of the leaves (*chlorophyll*[2]) and utilised in the photochemical reaction. For all other energy-consuming synthetic processes in the animal and vegetable organism the energy required must be supplied by *simultaneously occurring processes of degradation*, such as *respiration, fermentation* and *glycolysis* (see p. 233). This holds, for example, for the synthesis of glycogen from lactic acid, where the energy requirement is provided by the simultaneous oxidation of a certain proportion of the lactic acid to CO_2 and H_2O (Meyerhof).[3]

The *assimilation of nitrogen*, *i.e.* the fixation of atmospheric nitrogen by certain bacteria which occur in the soil (azotobacter) with production of protein from the ammonia which is believed to be the intermediate product, also requires the application of a considerable amount of energy. Here, too, the energy is supplied by the combustion of carbohydrate which, in the case of the nodule-forming bacteria of leguminous plants, is supplied by the host, the plant with which the bacteria live in symbiosis.[4]

Three great classes of naturally occurring organic substances supply the chief materials of which living cells are constructed, these classes being the most important constituents of the animal and vegetable organism. Further, these are the substances which undergo the greatest variety of transformations or processes of degradation. The classes are:

---

[1] Willstätter and Stoll, *Untersuchungen über die Assimilation der Kohlensäure* (Berlin, 1918).

[2] Willstätter and Stoll, *Untersuchungen über Chlorophyll* (Berlin, 1913).

[3] See, for example, Meyerhof, *Naturwiss.*, 1931, 19, 923; Lohmann in Oppenheimer's *Handbuch der Biochemie*, Supplementary Volume I, Part 2, p. 851 (Jena, 1933); Meyerhof, *Die chemische Vorgänge im Muskel* (Berlin, 1930).

[4] See Burk in *Ergebn. Enzymforsch*, 1934, 3, 23.

1. **The carbohydrates** (see p. 65).
2. **The proteins** (see p. 47).
3. **The lipins**, with which must be included, in addition to the true *fats*, the phosphatides, cerebrosides, sterols and carotenoid compounds (see p. 87).

The synthesis of carbohydrates and proteins from inorganic material is carried out exclusively by plants. Animals, as a rule, cannot synthesise the units (sugars, amino-acids) from which these substances are built up but can only produce more complex materials from them. Thus, for example, the animal organism breaks down to amino-acids or other simple degradation products the protein consumed as food and synthesises from these simpler compounds the proteins of the body.

The conversion of carbohydrates and fats into each other, on the other hand, is carried out in the animal as well as in the vegetable organism.

As a result of the classical work of Hopkins, Hofmeister, Stepp, Eijkmann and others, we know, however, that the animal organism cannot maintain itself indefinitely, when fed exclusively with pure carbohydrates, proteins and fats, even when the necessary amounts of mineral salts, small quantities of heavy metals and so on, are also provided. The organism requires, in addition, minute amounts of certain highly specific organic accessory factors, the *vitamins* which, in general, are produced only by plants but are indispensable to the animal organism, especially the growing organism, either in the synthesis of particular substances or else for the fulfilment of certain functions. The most important vitamins have now been isolated in the pure state and, in the case of some of them, the constitution has been wholly, or to a great extent, elucidated. Several of them, *e.g.* the water-soluble vitamin-B_2 (lactoflavin) and the antiscorbutic vitamin-C, have also been obtained synthetically (see p. 68).

All processes of degradation and, we may also suppose, most biological syntheses and transformations are stimulated by certain characteristic, highly specific and exceedingly sensitive *catalysts* known as *enzymes*,[1] which are produced by the living cell. Many of the reactions initiated by enzymes, like many non-enzymic catalytic reactions (*e.g.* the inversion of cane-sugar by H-ions), occur in homogeneous media. Others are surface (interface) reactions occurring within the cells and resembling, let us say, the reaction involved in the well-known synthesis of ammonia with contact catalysts.[2] The enzymes act in astonishingly small amounts and, consequently, occur in plant and animal organisms in almost inconceivably high dilution only. In no case has an enzyme yet been isolated in the pure state,[3] but experience acquired with some enzymes in recent years leads to certain conclusions, experimentally verified, concerning their nature. They consist of a characteristic, chemically well-defined " *active group* " united to a " *colloidal carrier* " of high molecular weight. Only when this special type of union persists do they exert their powerful catalytic action on the substance (" *substrate* ") which undergoes transformation.

Characteristic of all chemical processes occurring in the animal and vegetable organism is an extraordinarily delicate *regulatory mechanism*. The processes of growth, for example, and the utmost variety of function exhibited by the organs, are controlled by such mechanisms. In the higher animals the functions are partly regulated by the nervous system but, in the animal as well as in the vegetable kingdom, regulation by " chemical messengers ", the so-called *hormones*, is equally important. These are substances produced in certain parts of the organism (frequently as a result of some stimulus) and passed into the circulation (in plants into the capillaries), in order that they may exert their regulatory powers, generally in quite different parts. In this way they inhibit or stimulate chemical transformations or other processes. The hormones also, like the enzymes and vitamins, act in extremely minute amounts. Since they occur only in the smallest traces they are extraordinarily difficult to isolate but in several cases isolation has been successfully carried out. Some of them are relatively stable substances of low molecular weight allied to the vitamins. Others are very sensitive substances of high molecular weight similar, perhaps, to the enzymes (insulin, see p. 78).

---

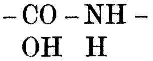
[1] The term " ferment " is also used but is less satisfactory.

[2] In this connexion see, for example, Thorpe's *Dictionary of Applied Chemistry*, 4th Ed., p. 333 (London, 1937).

[3] Some of the crystalline enzymes very recently obtained may have been pure.

The *degradations* which necessarily occur in the organism in order to provide energy usually begin with *hydrolyses.* All these hydrolyses proceed essentially in the same way: water is added on, and, as a result of the action of *hydrolytic enzymes,* cleavage of primary valencies occurs. Typical examples of such reactions are:

(*a*) Amongst *proteins,* cleavage of *peptide links*



$\underset{\text{OH}}{-\text{CO}}\underset{\text{H}}{-\text{NH}-}$

with production of carboxy- and amino-groups. Peptones, polypeptides and, finally, amino-acids are thus obtained. The enzymes which cause this kind of degradation are called *proteases* (see p. 103).

(*b*) Amongst *polysaccharides* cleavage of *glucoside links* between the various carbohydrate units. In this way starch and glycogen are converted by *amylases* or *diastases* into maltose, which is further degraded by *maltase* to glucose. In a similar way, as a result of the action of certain enzymes, fructose is produced, *e.g.* from inulin, xylose and galactose from xylans and galactans, and so on (see p. 121).

(*c*) *Fats* are converted by *lipases* or *esterases* into fatty acids and glycerol, and phosphatides, in the same way into fatty acids, choline and glycerophosphoric acid. The hydrolysis of phosphatides and other organic phosphorus compounds is brought about by a special group of esterases called phosphatases (see p. 136).

(*d*) *Nucleic acids* are broken down in several stages (polynucleotides, simple nucleotides) into phosphoric acid and nucleosides, and these, in their turn, are converted by nucleosidases into purines or pyrimidines and carbohydrates (see p. 80).

Generally, the hydrolyses are only preliminary steps in the actual energy-producing degradation and they are characterised, biologically, by the fact that they yield only extraordinarily little free energy. Consequently, many of these reactions are even reversible, the reversion being brought about by suitably altering the medium in which the process takes place.

The actual degradations, on the other hand, are of quite a different character. They produce deep-seated changes in the cells, the direction of the changes being towards the greatest degree of simplicity. Correspondingly, they produce much free energy and for the most part they are not reversible under ordinary conditions. Usually, these processes involve the *cleavage of carbon chains*; accordingly, they are termed *desmolytic* processes (from δεσμός, bond, and λύειν, to loosen) in contrast to hydrolytic processes. The enzymes concerned are called *desmolases*. The use of these terms was suggested by C. Neuberg and C. Oppenheimer.[1]

The final products of the *degradation of carbohydrates in the animal organism* are CO_2 and H_2O; thus, as in the case of combustion, the degradation is an oxidative process which constitutes a reversal of assimilation (" dissimilation "). Actually, since the time of Lavoisier, these and similar degradations have been regarded as species of slow combustion of foods similar to ordinary combustion at higher temperatures. Desmolysis, as it is understood in modern biochemistry and cell physiology, involves quite a different conception, namely, the conception of a series of *consecutive reactions* each of which is accompanied by a decrease in amount of energy. At each successive stage the degradation is carried only a little further and, after many intermediate stages, the end-products CO_2 and H_2O are finally reached.

It is remarkable and characteristic that it is only in the *last* stages of the degradation that *oxygen* is involved, whilst a series of phases, particularly those which occur first, proceeds without the intervention of oxygen.

In the early stages of the degradation of the hexoses free oxygen is not involved, hydrogen and oxygen being shifted from one part of the molecule to the other, and molecular groupings rich in hydrogen or poor in oxygen and poor in hydrogen or rich in oxygen are produced so that the carbon chain is broken. Thus, for example, the following equation represents the anaerobic production of lactic acid (glycolysis) in the animal body :

$$\underset{\text{Hexose}}{CHO.(CHOH)_4.CH_2OH} \longrightarrow \underset{\text{Lactic Acid}}{2CH_3.CHOH.COOH}$$

According to this equation two of the C-atoms are oxidised to the highest possible degree (carboxyl), whilst two others carry the maximal amount of hydrogen.

The *intervention of oxygen* (respiration) never occurs in such a way that the element is directly introduced into the molecule undergoing oxidation. Groups in the degradation products rich in hydrogen rather undergo alteration involving loss of hydrogen, *i.e.* they are dehydrogenated and this hydrogen eventually unites with oxygen yielding water. In this process the cell catalysts act, on the one hand, on the hydrogen of the oxidisable substances of the cell, increasing its reactivity (dehydrogenases), and, on the other hand, they activate the oxygen itself (oxidases).

---

[1] *Biochem. Z.*, 1925, **166**, 450.

Whilst in the higher animals the processes which occur in the absence of oxygen and those degradation processes (respiration) which involve consumption of oxygen interact, the energy-producing *metabolism* of many lower organisms, *e.g.* many *bacteria* and *yeasts*, is in the main not dependent on oxygen. Energy is produced in these organisms as a result of oxidation-reduction reactions of the same kind as those which occur, without the intervention of oxygen, in the early stages of carbohydrate breakdown in the higher organisms ; *e.g.*

1. $C_6H_{12}O_6 \longrightarrow 2C_3H_6O_3$
Lactic acid fermentation
and glycolysis

2. $C_6H_{12}O_6 \longrightarrow 2CO_2 + 2C_2H_5OH$
Alcoholic fermentation

The yield of energy in these processes which occur without the intervention of oxygen is much smaller than in true respiratory processes. Consequently, cells which live entirely anaerobically must, in general, transform great amounts of material in order to be able to meet their energy requirements.

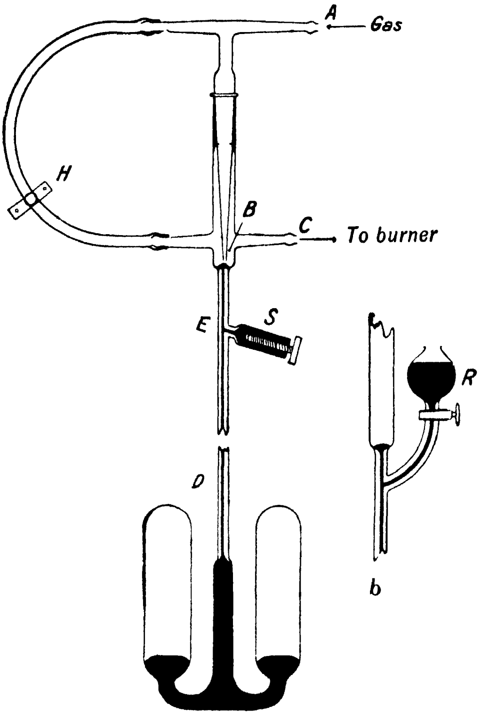
I. GENERAL TECHNIQUE

THE THERMOSTAT

In biochemical work (*e.g.* with enzymes, bacteria, sections of tissue and so on) the physico-chemical state of the external medium must be given much more attention than is generally necessary in the case of other chemical transformations. This is so because of the peculiarities of many physiologically important materials, their sensitivity to all external influences and the readiness with which they are transformed into other substances. Conditions which must be fixed, and, where necessary, kept constant, at least in all experiments in which measurements are made, are, temperature, hydrogen-ion concentration, concentration of other ions and reactants present, occasionally gas pressure, oxidation-reduction potential and so forth. It should be a fundamental rule that experiments are comparable only when carried out under the same external conditions.

The chief means of maintaining constant temperature are incubators (or constant-temperature rooms) and thermostats. The disadvantage of incubators and constant-temperature rooms is, that vessels placed in them assume the temperature concerned relatively slowly only. Consequently, such arrangements are more adapted for experiments of long duration and, as far as temperature is concerned, for experiments which are not particularly delicate (*e.g.* propagation of bacteria and the like). In all other cases it is more appropriate to use thermostats.

Thermostats are constructed from vessels of large capacity and are provided with stirring apparatus and thermo-regulators so that the water in the vessel is automatically maintained at the constant temperature desired. Large enamelled pails (capacity, say, 7–15 litres) suffice for many purposes. Rectangular vessels are more expensive but, in general, more convenient; they are to be preferred when it is necessary to use large instruments, for example, shakers and the like. In order to diminish the loss of heat to the surroundings the vessels are wrapped in asbestos sheets or in felt. Ready-made thermostats, *e.g.* the well-equipped thermostats used for respiration experiments according to Warburg's method, can be purchased but are not cheap. They can be constructed without difficulty by the student with the help of the laboratory mechanic, and, in any case, this is the simpler way.



FIGS. 1*a* and 1*b*

Thermostats are heated either electrically or with gas. Electric heating is more expensive to install and use but is to be preferred when the apparatus is to be continually in use. If gas-heated thermostats are to be left unattended, overnight, for example, the gas must be supplied to the burners in iron or lead pipes (not rubber tubes).

Figs. 1*a* and 1*b* show two useful forms of temperature regulator for use with gas. The gas enters the instrument at *A* and passes by way of *B* and *C* to the burner. The vessel *D* which contains toluene or amyl alcohol is completely immersed in the water of the thermostat. When the temperature of this water exceeds a certain value the gas current is cut off at *B* by the rising of the mercury in *E*. In order, at this stage, to prevent complete extinction of the flame a reduced current of gas may be allowed to flow to the burner by way of the by-pass in which the current is checked by means of the screw-clip *H*. The position of the mercury and, consequently, the temperature at which the gas current in *B* is cut off are adjusted by means of the regulating screw *S* (Fig. 1*a*) or by running in mercury from the storage bulb *R* (Fig. 1*b*) which is provided with a cock.

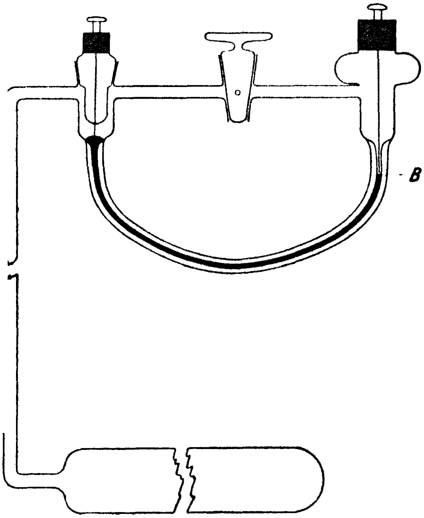


FIG. 2

The device for electrical regulation of temperature consists of a heating element (immersion heater or special incandescent globe for thermostats), the actual temperature regulator (Fig. 2) and an interrupter (see-saw) for the heating current (Fig. 3). For the rest the action of this regulator is exactly similar to that of the regulator for gas: when the mercury rises in *B* (Fig. 2) a secondary circuit is completed and this current, in its turn, switches off the main current by means of the interrupter. When the mercury then falls again the secondary circuit is broken, the see-saw moves into its original position, the main circuit is again completed and so the heating begins again. The interrupter requires a potential of 6–8 volts. Higher potentials are undesirable because they lead to the production of larger sparks in the regulator at *B* and the glass may thus be cracked. The usual light or power current, of course, serves for heating the thermostat. Two common forms of thermostat are shown diagrammatically in Figs. 4*a* and 4*b*. In *a* the interrupter is connected to an accumulator and in *b* both circuits are connected to the lighting circuit, the secondary current which passes through the magnets and the thermoregulator being reduced to the level just required for actuating the interrupter by adjustment of the sliding resistance W_2. The current which passes through the resistance W_1 (which must be large in comparison with W_2), the resistance W_2 and the

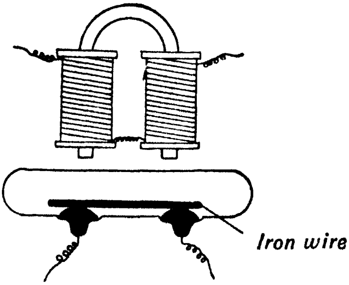


FIG 3

continuously heated element H_2, must be just sufficient to maintain a temperature just below that at which it is desired to maintain the thermostat.

It is easily possible (even in improvised thermostats) to keep the temperature constant to within ± 1°, this being sufficient for most of the experiments afterwards described. If the dimensions of the various parts are suitably chosen and the apparatus is carefully designed it is possible, without great difficulty, to maintain a temperature constant to within approximately 0·01°, especially when electrical heating is adopted.

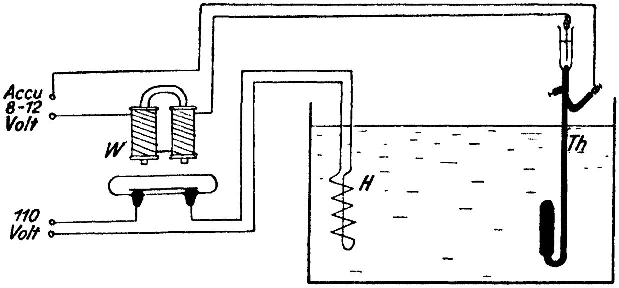


FIG. 4a

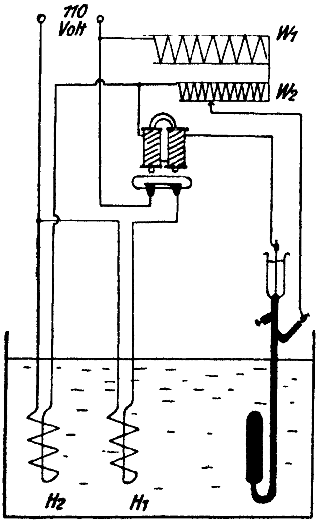


FIG. 4b

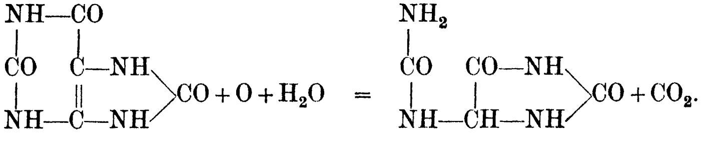
1. MEASUREMENT AND ADJUSTMENT OF p_H

Not less important for the course of biochemical processes is well-defined *reaction* of the medium. In particular, most processes involving enzymes are notable for the demands which they make in this connexion. Every enzyme is characterised by a relatively narrowly limited zone of reaction. Within this zone it exerts its maximal activity (*optimum of reaction*). Outside this zone the extent of the change which occurs decreases greatly and when the deviation from the optimum is sufficiently great the enzymic reaction may cease completely. Thus, for example, the saccharification of starch (see p. 133) caused by the amylase of the pancreas requires a p_H of 6·8 for optimal effect. Invertase, the enzyme which converts sucrose into glucose and fructose, is most active at p_H 4·5–5·0, whilst the proteolytic enzyme trypsin confines its action to a faintly alkaline medium with p_H about 8·9.

Not infrequently, however, such statements are to be regarded as rough guides in investigations with enzymes. It has been found, indeed, that the dependence of an enzymic reaction on p_H is affected in a manner often not easy to understand, by the *origin* and *degree of purity of the enzymic material* used, by the nature of *activators* and *inhibitors* which may be present and even, in many cases, by the choice of *substrate*. Aminopolypeptidase, for example, appears to vary according to its origin since polypeptides are best hydrolysed by vegetable material (yeast) at p_H 7 but maximal hydrolysis is caused by animal material (intestinal mucous membrane) at p_H 8. Possibly this difference in behaviour is also connected with the presence of different kinds of impurity. Thus, for example, the lipases of the digestive secretions of animals differ in the positions of their optima of activity, that for gastric lipase being in the acid, that for pancreatic lipase in the alkaline zone. Progressive purification of the gastric lipase, however, shifts its optimum of reaction towards the alkaline zone, *i.e.* towards identity with the pancreatic enzyme. The discovery that certain substances which accompany enzymes sometimes activate, sometimes inhibit, an enzymic reaction—according, indeed, to the p_H which prevails—is an important factor in the understanding of such facts. As an example of the fact that the substrate also may occasionally displace the p_H optimum of an enzyme it may be mentioned that gelatin is best hydrolysed by yeast-proteinase at p_H 5·0, whilst neutral reaction has proved to be most favourable with the substrates fibrin and edestin.

The maintenance of an appropriate p_H is equally necessary for the *purification* and *fractionation* of enzymes and similar natural products and, consequently, for example, for the purification of enzymes by *adsorption* and subsequent *elution*.

Finally, the systematic study of the dependence of enzymic reactions on p_H often throws *light* on the *mechanism* of biochemical and physiological processes. This is especially so when it is possible by changing the p_H of a naturally occurring mixture of enzymes to maintain the activity of only one of the components. The conversion of uric acid into allantoin[1] by "uricase" requires the co-operation of at least two (possibly three) enzymes. The first of these brings about the introduction, at p_H 8·9, of O and H_2O into the uric acid molecule, whilst a second enzyme, at p_H 9·9, brings about the decarboxylation of the intermediate product, the structure of which has not yet been elucidated.



Thus, since it is possible to separate the two phases of uricolysis by adjusting the concentration of H-ions, the actions of the two enzymes can be distinguished.

DEFINITIONS

Introductory.—The H- or OH-ion content of an acid or alkali cannot be directly deduced because, first, *ionic association* tends to occur as a result of the action of interionic forces in *strong electrolytes* so that the number of free mobile H- and OH-ions, which alone determines the reaction, is reduced (the conception of H-activity and of "*apparent dissociation*"), and, second, *weak electrolytes* are actually only *partly dissociated* in solution. The bridge which leads from the total concentration to the concentration of free H- and OH-ions is in the one case the *activity coefficient*, in the other, Ostwald's *dilution law*. But, as a result of circumstances which will not be further examined here, these give only a relatively crude and usually quite inadequate approximation to the reality. Consequently, a series of practical *methods* which avoid these difficulties has been worked out for measuring p_H.

In the study of enzymes the p_H must not only be *adjusted* but must also be *maintained*. For slight contamination of the reaction medium by atmospheric carbon dioxide, by alkali from glass, or by products resulting from the activity of the enzymes, may cause an appreciable deviation from the optimum for the reaction and, consequently, a disturbance of the sensitive enzymic reaction.

In accordance with S. P. L. Sørensen's suggestion the reaction of a solution is expressed by the *negative logarithm* (base 10) *of the hydrogen-ion concentration* :

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[1] Felix, Scheel and Schuler, *Z. physiol. Chem.*, 1929, **180**, 90.

$$p_H = -\log [H^{\cdot}].$$

A solution having $[H^{\cdot}] = 10^{-1}$ has, consequently, $p_H = 1$, whilst a solution having $[H^{\cdot}] = 10^{-2}$ has $p_H = 2$, and 0 is the p_H of a solution having $[H^{\cdot}] = 10^0 = 1$. Thus the greater the p_H the smaller is $[H^{\cdot}]$. $[H^{\cdot}]$ is converted without difficulty into p_H when it is remembered that, by definition, $[H^{\cdot}]$ is to be expressed as a logarithm (base 10) and the value obtained multiplied by -1. Conversely, p_H is converted into $[H^{\cdot}]$ by multiplying by -1 and taking the anti-logarithm, *e.g.* p_H 7·70 :

$$[H^{\cdot}] = 10^{-7 \cdot 7} = 10^{0 \cdot 30} \times 10^{-8} = 2.10^{-8}.$$[1]

In the same way—and this is the advantage of this process—the *strength of alkalis* may also be simply expressed, when it is remembered that the product of the concentrations of the ions of water is a constant :

$$[H^{\cdot}].[OH'] = 10^{-14 \cdot 14} = K[H_2O] \quad (18^\circ)$$
$$\log [H^{\cdot}] + \log [OH'] = -14 \cdot 14$$
$$p_H + p_{OH} = 14 \cdot 14.$$

Thus, for example, when $[OH']$ is 10^{-3} then $[H^{\cdot}]$ is $10^{-11\,14}$ and p_H is 11·14. The p_H at the neutral point is 7·07 since here $[H^{\cdot}] = [OH']$. All solutions having $p_H < 7 \cdot 07$ are acid, all those having greater p_H are alkaline.

BUFFERS

By using *buffer solutions*, solutions, namely, which contain weak acids or bases, and their salts in varying proportions, one can maintain systems at *sharply defined reaction*. Further, such solutions are characterised by the fact that they can fix limited amounts of H- and OH-ions which may enter the system from without or which may be produced internally during some process, so that the reaction of the buffer solution is not appreciably altered. The ratio of the concentrations of the components of the buffer solution is altered but this alteration is of no consequence. As buffers one uses, for example, the mixtures suggested by Sørensen,[2] glycine + hydrochloric acid (acid zone), sodium citrate + hydrochloric acid or sodium hydroxide, alkali dihydrogen phosphate + di-alkali hydrogen phosphate, borate + sodium hydroxide. These substances are used in the following concentrations :

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[1] The logarithm of $10^{0 \cdot 30}$ is 0·30. log 10 = 0·30. of which the anti-logarithm is 2. Consequently, each increase of p_H by 0·3 halves the hydrogen-ion concentration.

[2] Sørensen, *Biochem. Z.*, 1909, 21, 131.

Hydrochloric acid : 0·1 *N*-HCl.
Sodium hydroxide solution : 0·1 *N*-NaOH.[1]
Glycine 0·1 *N* : 7·505 g. of glycine + 5·85 g. of NaCl in 1 litre of water.
Potassium dihydrogen phosphate 0·0067*M* : 9·078 g. of KH_2PO_4 in 1 litre of water.
Disodium hydrogen phosphate 0·0067*M* : 11·876 g. of $Na_2HPO_4.2H_2O$ in 1 litre of water.
Disodium hydrogen citrate 0·1*M* : 21·008 g. of crystalline citric acid dissolved in 200 c.c. of *N*-NaOH and diluted to 1 litre.
Borate : 12·404 g. of boric acid dissolved in 100 c.c. of *N*-NaOH and diluted to 1 litre.

These solutions are prepared with boiled-out water free from CO_2 and are stored in bottles provided with soda-lime tubes.

The hydrogen-ion concentrations of such mixtures are accurately determined by means of the hydrogen electrode or other standard electrode (cf. p. 24) and are plotted : in the graph shown opposite the ordinates give the composition for each 10 c.c. of solution of the p_H concerned, and the abcissae the p_H of the various mixtures. The ordinate value 0, for example, corresponds to pure 0·1 *N*-HCl or 0·1 *N*-NaOH and the ordinate value 10 to pure glycine. If a few examples are given it will be easy to understand how the curves are used :

p_H 2·5	6·67 c.c. of glycine + 3·33 c.c. of HCl
p_H 4·0	5·62 c.c. of citrate + 4·38 c.c. of HCl
p_H 7·0	6·10 c.c. of Na_2HPO_4 + 3·90 c.c. of KH_2PO_4
p_H 10·0	5·94 c.c. of borate + 4·06 c.c. of NaOH.

The buffering action naturally persists as long as both the *active constituents* remain present together in the solution. The buffering power of the system ceases as soon as one component is completely consumed. Hence, we have the rule: when making buffer solutions always search for *steep parts of the curve,* since gently sloping or horizontal parts represent p_H zones in which, because one component predominates greatly, there is no appreciable buffering power. In the curves the mixtures to be avoided are indicated by dotted lines.

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[1] Preparation of sodium hydroxide solution free from carbonate : dissolve 250 g. of sodium hydroxide in 300 c.c. of water in a narrow, stoppered, cylinder. Sodium carbonate is practically insoluble in sodium hydroxide solution of this concentration and, consequently, sinks to the bottom in the course of a few days. Draw off the clear solution and dilute it to normality 0·1 with CO_2-free water.

Other than 0·1 *N*-solutions can also be used for the mixtures described above without important alteration. Only the buffering power is thus altered: it increases with increasing concentration and conversely.

L. Michaelis has added some other mixtures to those of Sørensen.[1]

Mixing Proportions	0·1 *N*-NH_4Cl 0·1 *N*-NH_3	0·1 *N*-Tatraric Acid 0·1 *N*-Na Tatrate	0·1 *N*-Lactic Acid 0·1 *N*-Na Lactate	0·1 *N*-Na Acetate 0·1 *N*-Acetic Acid
9·7 : 0·3	p_H = 8·0	p_H = 1·4	p_H = 2·3	p_H = 3·19
9·4 : 0·6	,, = 8·3	,, = 1·7	,, = 2·61	,, = 3·3
8·9 : 1·1	,, = 8·58	,, = 2·0	,, = 2·9	,, = 3·8
8·0 : 2·0	,, = 8·89	,, = 2·4	,, = 3·2	,, = 4·1
6·67 : 3·33	,, = 9·19	,, = 2·7	,, = 3·5	,, = 4·4
5·0 : 5·0	,, = 9·5	,, = 3·0	,, = 3·8	,, = 4·7
3·33 : 6·67	,, = 9·8	,, = 3·3	,, = 4·17	,, = 5·0
2·0 : 8·0	,, = 10·1	,, = 3·6	,, = 4·45	,, = 5·3
1·1 : 8·9	,, = 10·4	,, = 3·8	,, = 4·7	,, = 5·6
0·6 : 9·4	,, = 10·7	,, = 4·2	,, = 5·0	,, = 5·9
0·3 : 9·7	,, = 11·0	,, = 4·5	,, = 5·3	,, = 6·22

The values for the p_H shown on the curves and table above hold, in general, for *room temperature.* The reaction undergoes *slight alteration* if *other temperatures* are employed.

It may be pointed out that, in nature, the numerous enzymic processes of the vegetable and animal organism are likewise facilitated by means of a series of physiological buffering systems, *e.g.* the mixture of phosphates in the fluids of plants and the haemoglobin and carbonate-hydrogen carbonate system in the blood.

MEASUREMENT OF p_H WITH INDICATORS

In most cases p_H can be determined with sufficient accuracy but without the use of special apparatus by means of *indicators.*

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[1] Finally, there are the mixtures HCl – KCl, HCl – hydrogen phthalate, NaOH – hydrogen phthalate, NaOH – KH_2PO_4, NaOH – boric acid – KCl, $Na_2B_4O_7$ – boric acid, HCl – Na_2CO_3, as well as some citrate buffers, details about which are given in the original papers: Clark and Lubs, *J. Bact.*, 1917, **2**, 1, 109, 191; Palitzsch, *Biochem. Z.*, 1915, **70**, 341; Kolthoff and Vleeschhouwer, *Biochem. Z.*, 1926, **179**, 410; 1927, **183**, 444; McIlvaine, *J. Biol, Chem.*, 1921, **49**, 183. See also Oppenheimer and Pincussen, *Die Methodik der Fermente*, vol. 3 (Leipzig, 1929).

These are *weak acids or bases* or their salts and *according to the reaction which prevails,* they exhibit a *variety of colours.* According to W. Ostwald these colour changes depend on an increase or decrease in the degree of dissociation of the coloured indicators, whilst A. Hantzsch was able to show it to be probable that they are connected, in many cases, with the shifting of a mobile H-atom and displacement of double bonds (benzenoid-quinonoid system). The changes may also be mathematically expressed, on the basis of the law of mass action. For example, the following expression holds for an indicator acid :

$$\frac{[H^{\cdot}].[A']}{[HA]} = k.$$

Increase in the H-ion concentration causes decrease in the extent of dissociation and development of the colour of the undissociated molecule HA, whilst decrease in the H-ion concentration causes extensive dissociation of the coloured molecule and, hence, causes the development, in the solution, of the colour of the anion A′. Between the two extremes of colour there is an intermediate zone—the actual zone of colour change in fact—in which the undissociated molecules and the coloured anions are present together in com-

Common Name	Systematic Name	Zone of Colour Change	Colour in	
			Acid Solution	Alkaline Solution
Thymol blue *	Thymolsulphonephthalein	1·2– 2·8	red	yellow
	Benzenesulphonic acid-*p*-azo-benzylaniline	1·9– 3·3	red	yellow
Bromophenol blue	Tetrabromophenolsulphonephthalein	3·0– 4·6	yellow	blue
Methyl orange	*p*-Dimethylamino-azobenzenesulphonic acid	3·1– 4·4	red	yellow
Methyl red	*p*-Dimethylamino-azobenzene-*o*-carboxylic acid	4·0– 6·0	red	yellow
Bromocresol purple	Dibromo-*o*-cresolsulphonephthalein	5·2– 6·8	yellow	violet
Bromothymol blue	Dibromothymolsulphonephthalein	6·0– 7·6	yellow	blue
Phenol red	Phenolsulphonephthalein	6·8– 8·4	yellow	red
Naphtholphthalein	α-Naphtholphthalein	7·3– 8·7	faint pink	blue
Cresol red	*o*-Cresolsulphonephthalein	7·2– 8·8	yellow	red
Cresolphthalein	*o*-Cresolphthalein	8·2– 9·8	colourless	red
Phenolphthalein	..	8·3–10·0	colourless	red
Alizarin yellow R	Nitrobenzene-*p*-azosalicylic acid	10·3–11·7	yellow	red

* Thymol blue exhibits, in addition, a second zone of colour change between 8·0 and 9·6 (yellow—blue). Cresol red and phenol red become pink at p_H less than 2 besides changing in the zone shown in the table.

parable amounts. Then, according to the proportions in which the components are present, the solution exhibits varying shades intermediate between the colour of the strongly acid and that of the strongly alkaline solution.

The change of colour, however, occurs with the various indicators at quite different p_H and also extends over intervals of various magnitudes. These intervals have been systematically measured for a great number of dyes. In the table on page 16 the chief indicators recommended by Clark and Lubs are listed.[1]

If a few drops of the indicators listed are added to a sample of a solution of unknown H-ion concentration one of them will then be in its zone of colour change and will exhibit an intermediate colour. In this way the p_H is determined to a first approximation, since it is to be sought in the zone between the two extreme points of colour change of this indicator. The method may be rendered more delicate by preparing buffer solutions having H-ion concentrations differing from each other by regular amounts (as shown by their different shades of colour) and all lying within the determined zone. It should be borne in mind that, if a definitive statement of p_H is to be made, two such solutions must always be found for comparison in order that the shade of colour of the unknown solution will be intermediate between those of the two standards.

Experiment.—Determine the p_H of 0·01 N-acetic acid, of spring water and of freshly boiled-out distilled water. In addition, determine the p_H of saliva or blood-serum.

Procedure.—Steam for some time the test-tubes to be used, wash them with distilled water and dry them.

1. Observe the behaviour of indicators on addition of acid and alkali. With thymol blue add first to the acid and then to the alkali, with phenol red and cresol red add to the acid potassium dihydrogen phosphate ($p_H = 4{\cdot}5$).[2]

2. Prepare a series of test-tubes each containing 1 c.c. of the liquid to be examined, add to each 1 drop of indicator solution diluted in varying degrees and so determine which is the appropriate indicator. If in doubt note which indicators give a markedly "acid" and which a markedly "alkaline" colour. The indicators showing intermediate colours must fall between the two groups. In certain cases there will be some overlapping in the zones of colour change and thus far-reaching delimitation becomes possible. Choose

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[1] *J. Bact.*, 1917, 2, 1, 109, 191.

[2] See footnote to table on opposite page.

that indicator for which the H-ion concentration, as far as it can be estimated in the manner indicated, lies in the middle of the zone of colour change.

3. Determine the amount of indicator necessary by adding to 10 c.c. of the liquid under examination the number of drops required to produce a moderately dark shade of colour.

4. Prepare several buffer solutions (10 c.c. of each) having p_H within the zone of colour change of the indicator concerned (if the zone has been narrowly delimited prepare buffer solutions accordingly) each solution differing in p_H from the next by 0·2 unit and place them on a sheet of milk-glass. To each of the solutions add the same number of drops of the indicator (the number determined as described above) so that a series of intermediate colours lying within the zone concerned is obtained. Against the white background it is then easy to fit into its place in the series the tube with the solution having the unknown H-ion concentration. Place the tube on the sheet of glass and look down from above. In this way even slight differences which may still persist can be observed very easily. After the delimitation has been carried so far prepare, if necessary, three further intermediate solutions, differing in p_H by 0·05 unit and compare them with unknown solution. In this way the p_H may be determined still more accurately—to within about 0·05 unit in the most favourable cases.

Coloured and Turbid Solutions.—The colorimetric method cannot be applied to strongly coloured solutions. When the colour is less pronounced, attempt to attain the shade of colour desired by adding a suitable dye to the solutions for comparison before adding the indicator.

When using turbid solutions, *e.g.* when examining colloidal proteins or colonies of suspended bacteria, make the standard solutions equally turbid, as far as this may be possible, by adding, say, suspension of finely divided quartz or colloidal barium sulphate. Use for this purpose exactly neutral suspensions only and not amphoteric hydroxides which would alter the p_H of the standard solutions.

Other Procedure.—Arrange three pairs of test-tubes, all of the same dimensions, in a bored wooden block provided with a white background in such a way that the observer sees one tube of each pair behind the other.

Into each of the tubes at the back pour 10 c.c. of the turbid or coloured solution to be examined and, in addition, pour into the middle tube of the three a sufficient number of drops of a suitable indicator to produce a distinctly recognisable colour in the zone of colour change. Into the front tube of the middle pair pour 10 c.c. of distilled water and into each of the tubes on each side 10 c.c. of a suitable buffer solution together with the same amount of indicator as was added to the unknown solution. Alter the composition of this mixture until, on looking through the middle pair of tubes (turbid solution coloured with indicator + water), the appearance observed is either as exactly as possible the same as that seen on looking through one of the pairs at the side (turbid solution + buffer solution coloured with indicator) or may be fitted in between the pairs at the sides in respect of the shade of colour.

Determine the p_H of a 2 per cent gelatin and a 2 per cent peptone solution.

Error due to Protein.—Determine the zone of colour change of Congo red. Now prepare a citrate mixture having p_H 3·5 and an albumin solution having about the same reaction (indicator methyl orange). Add to one sample of the buffer solution methyl orange, to another Congo red, and then pour into each sample a few c.c. of the albumin solution. The shade of colour of the Congo red is greatly altered by the addition of the albumin, whereas that of the methyl orange remains practically unaltered.

If, in a solution to be examined, substances occur which bind the indicator or its ions by adsorption or salt formation, the equilibrium between indicator and H-ions, which is the basis of the colorimetric method, will be disturbed. In such a solution an indicator may exhibit an intensity or shade of colour distinctly different from that exhibited in a solution free from such substances. The effect, which is often troublesome, particularly in measuring the p_H of proteins, is called the protein error.

Error due to Salts.—Prepare an $NH_4Cl - NH_3$ and a borate buffer having the same p_H and add phenolphthalein to each. When common salt is added, the colour becomes deeper in the $NH_4Cl - NH_3$ buffer but lighter in the borate buffer.

Salts frequently alter the activity of the H-ions and, hence, also the reaction of the solution. The magnitude and direction of the effects may vary considerably in systems having different compositions but originally the same reaction and appreciable deviations may result.

The Colloid Membrane Colorimeter.[1]—The indicator method is considerably simplified by the use of the colloid membrane colorimeter. This is provided with several series of cellulose membranes coloured with Clarke and Lubs indicators. A suitable membrane is immersed for a definite period, measured with an hour-glass, in the solution under examination. The collection of membranes is provided with several scales of standard colours for comparison, with intervals of p_H 0·2, so that required p_H can be easily determined to within about 0·1–0·2 p_H units within a few minutes. Buffer solutions for comparison are thus rendered superfluous. An important point is that the membranes offer little resistance to the diffusion of aqueous solutions and so, especially, to H- and OH-ions, whilst they very firmly retain the indicators. The special advantage of the method is that it can be applied to extremely turbid solutions, to pulped organs and to other such suspensions. However, under certain conditions, diffusion phenomena give rise to errors of considerable magnitude.

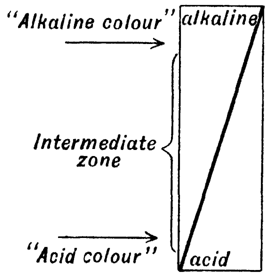

FIG. 5

The Double-Wedge Colorimeter of Bjerrum and Arrhenius.—This apparatus also makes possible the colorimetric determination of the p_H of liquids, even of coloured liquids, without the use of buffer solutions for comparison. A device consisting of two wedge-shaped vessels, containing one the " acid " the other the " alkaline " colour of a suitable indicator, is used. By sliding one of the wedges over the other all intermediate colours are automatically produced (see Fig. 5). The solution under examination, likewise containing indicator, is moved by means of a sliding carrier to the position where the colours match and the p_H value is found in a table.[2]

MEASUREMENT OF p_H BY MEANS OF CONCENTRATION CHAINS[3]

Hydrogen-ion concentration can also be determined, not so simply but in many cases with greater certainty, by means of *concentration chains.*

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[1] Wulff, *Kolloid-Z.*, 1926, **40**, 341; *Chem.-Ztg.*, 1926, **50**, 732; *Chem. Fabr.*, 1933, **6**, 441.

[2] For a precise colorimetric method of determining p_H see Du Rietz and Hähnel, *Svensk Kem. Tidskr.*, 1937, **49**, 284.

[3] For measurement of oxidation-reduction potential see page 173.

The procedure is best exemplified by the genuine concentration chains which consist of solutions containing *different amounts of the ion which determines the potential.* These solutions are in contact with each other and into them dip two *electrodes capable of acting reversibly.* In such a system there is generated an electromotive force which is calculated by means of the following expression worked out by Nernst :

$$E' = \frac{R.T}{n.F} ln \frac{c_1}{c_2} = 0{\cdot}4343 \frac{R.T}{n.F} \log \frac{c_1}{c_2},$$

where c_1 and c_2 are the concentrations of the ions concerned in the two solutions,[1] R is the gas constant, T is the absolute temperature, n is the valency of the ion concerned and F the number of coulombs (96500) carried by a univalent gram-ion. The constant 0·4343 R/F is equal to 0·0001983 when E' is expressed in volts so that, at room temperature ($T = 291°$), when the ratio of concentrations is $c_1/c_2 = 10$.

$$E' = \frac{0{\cdot}0001983.291.\log 10}{n} \text{ volt} = \frac{0{\cdot}0577}{n} \text{ volt.}$$

If then two solutions, say, one of unknown and one of known hydrogen-ion concentration, are connected in the appropriate manner, the ratio of the concentrations of H-ions, the hydrogen-ion concentration and, hence, the unknown p_H can be deduced from the *measured electromotive force*, provided that suitable reversible electrodes are used.

Now it has been found that such an electrode is formed when a strip of *platinum foil* which dips into the solution is *swept by a current of hydrogen.* The gas dissolves in the metal and, as regards electromotive force, behaves towards the solution exactly as Cu, for example, behaves to $CuSO_4$: it has a solution pressure, dependent on the partial pressure of the H_2 which prevails round the electrode at the time of measurement. Opposed to this solution pressure is the osmotic pressure with which is associated the pressure exerted by the H-ions which tend to leave the electrode. The resulting equilibrium between these two forces establishes an electrode potential which, however, requires to be related to some suitable standard value. *By definition* the value 0 is attributed to such a " single electrode potential " when H_2 at atmospheric pressure is in reversible equilibrium with a solution having normality 1[1] (this refers to H-ions) Pt electrodes being used (Reference electrode: the " normal hydrogen electrode ").

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[1] c_1 is chosen for a known concentration or, more accurately, activity, which is always used for the standard electrode.

Hence, if the value $c_1 = 1$ is given to the reference electrode then, since n is also equal to 1,

$$E' = 0{\cdot}0577 \log \frac{1}{c_2} \text{ volt.}$$

If now we let E be the absolute magnitude but take into account also the sign according to the potential which the electrode in the solution to be examined acquires, then, since c_2 is almost always less than 1 and so E negative,

$$E = -0{\cdot}0577 \log \frac{1}{c_2} \text{ volt} = 0{\cdot}0577 \log c_2 \text{ volt},$$

and therefore $$\log c_2 = \frac{E}{0{\cdot}0577},$$

and $$p_{\text{H}} = -\log c_2 = -\frac{E}{0{\cdot}0577}.$$

The requirement of reversibility is also met by an electrode with which H_2 is passed into an *alkali*. In this case, of course, the solution has $p_{\text{H}} > 7{\cdot}07$. For when the ionic product for water is taken into account it is at once possible to deduce for such a solution, the concentration of the ions which determine the potential (cf. p. 13).

Diffusion Potentials.—In addition to the electrode potentials concerned in the measurement of the actual p_{H} there are usually established also, at the *point of contact* of the liquids which surround the two electrodes, the so-called *diffusion or liquid potentials.* In particular these are always set up when two solutions of different composition, or two of the same composition but of different concentrations, are in contact, since the ions have different rates of diffusion. These potential differences have to be added (the sign being taken into account) to the electrode potentials. When a determination is being made they are not considered and, consequently, a corresponding correction must be subtracted from the measured E.M.F. But if the two solutions are not in direct contact, but are separated by saturated potassium chloride solution as intermediate liquid, the establishment of such potentials is, to a large extent, prevented and no correction need be applied.

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[1] This means H-activity = 1. In practice, this value is attained by using $2N\text{-}H_2SO_4$.

Vessel for Hydrogen Electrode.—A vessel of the form shown in Fig. 6 may be used. It is assembled in the following way. Wash the Pt electrode[1] with distilled water, dry it with absolute alcohol, fix it in position by means of an air-tight stopper and connect the vessel with the hydrogen generator by way of the tube *W*, the screw-clip *K* being closed. Dip the tube *S* into a beaker containing the solution under examination (or H_2SO_4), open the cock *H* and apply suction at *A*, so that liquid is drawn in until about half of the Pt foil is immersed (there must be no bubbles in the tube between *H* and *S*). Close *H*, open *K* and pass in hydrogen (washed by passage through $KMnO_4$ solution and alkaline pyrogallol solution) at such a rate that the individual bubbles can just be counted conveniently. From a pipette run into *A* some water to act as a seal, which prevents the entrance of air. Dip the tube *S* into KCl-solution which serves as intermediate liquid. Do not grease the cock *H* but moisten it all the way round by repeated turning. The film of liquid thus produced between the ground surfaces is a sufficiently good conductor when the cock is closed and detrimental diffusion is prevented. Wait until equilibrium is established (this frequently requires a long time).

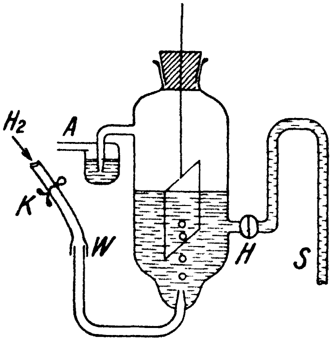

FIG. 6

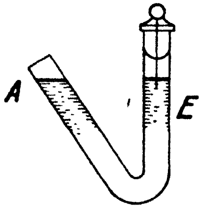

FIG. 7

U-Tube Electrodes in Stationary Hydrogen Atmosphere.—U-Tubes of the form shown in Fig. 7 are also used. Pour the liquid in such a way that no air bubbles remain in the leg *E* containing the electrode. Through a glass capillary tube pushed into *A*, until it reaches the bend, pass into the electrode leg just sufficient bubbles of H_2 to leave the tip of the Pt wire just dipping into the liquid. Connect with the electrode to be compared by means of a small tube of agar, prepared by laying a bent tube in a saturated KCl-solution in which has been dissolved by heating 3 per cent of agar, and leaving to cool.

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[1] Coated with platinum black. Re-coat from time to time. In this connexion see Ostwald-Luther, *Hand- und Hilfsbuch zur Ausführung physiko-chemischer Messungen*, 5th Ed., p. 186 (Leipzig, 1931).

In practice, other reference electrodes, such as the "*normal-calomel electrode*" or the saturated calomel electrode, are generally used instead of the normal hydrogen electrode. The normal calomel electrode, when connected with the normal hydrogen electrode, exhibits a positive potential of 0·284 volt and, consequently, E_1, the electromotive force registered when a solution under examination is connected to the calomel electrode, is less than the true value by this amount. In the calculation this must be taken into account

$$E = E_1 + 0{\cdot}284 \text{ volt},$$

and

$$p_{\mathrm{H}} = \frac{-E_1 + 0{\cdot}284}{0{\cdot}0577}.$$

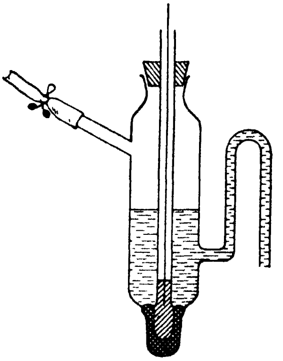

FIG. 8

The Normal Calomel Electrode (see Fig. 8).—Pour into the constricted part of the vessel mercury free from base metals until a layer 0·75 cm. deep is formed, and cover with a thin layer of powdered calomel. Make the vessel air-tight by closing with a rubber stopper carrying a glass tube through which passes a carbon electrode for the current. Adjust this electrode so that the tip, which projects from the glass tube, is completely immersed in the mercury. Then draw in by suction N-KCl solution until the vessel is half filled, having previously saturated the solution with calomel by shaking for at least two hours with that salt. Keep the bent side tube, even when not in use, immersed in a beaker of this solution and, also, always use the solution as intermediate liquid when measurements are being made against other electrodes.

Together with this apparatus a platinum-hydrogen electrode which dips into the solution under examination must be used but if the *quinhydrone electrode* is employed no hydrogen electrode is required. Quinhydrone, an addition compound of quinol and quinone, decomposes into its two components in aqueous solution. As a result of the oxidation-reduction potential set up at a polished Pt electrode such a quinhydrone electrode

$$C_6H_4(OH)_2 \rightleftarrows C_6H_4O_2 + 2H^{\cdot} + 2\ominus$$

behaves like a hydrogen electrode with a very low gas-pressure (cf. p. 21).

When connected with a solution having the same H-ion concentration but containing no quinhydrone the quinhydrone electrode at 18° acquires a positive charge of 0·7044 volt and, consequently, E.M.F.'s measured with this electrode are too high by that amount. If, as is customary, the quinhydrone electrode is compared with the calomel electrode, the additional correction indicated above must be applied :

$$E = E_1 + 0{\cdot}284 - 0{\cdot}7044 \text{ volt.}$$

and

$$p_{\mathrm{H}} = -\frac{E_1 + 0{\cdot}284 - 0{\cdot}7044}{0{\cdot}0577}.$$

In practice, the quinhydrone electrode is prepared by pouring a saturated alcoholic solution of the substance into the solution under examination. The aqueous phase becomes supersaturated and part of the quinhydrone is precipitated. The quinhydrone electrode can be used in acid and slightly alkaline media only. When the p_{H} exceeds 8 it cannot be used. Moreover, inaccurate results are produced by electrolytes but the errors thus caused can be eliminated by saturation with the products of decomposition as well as with quinhydrone (cf. Ostwald-Luther).

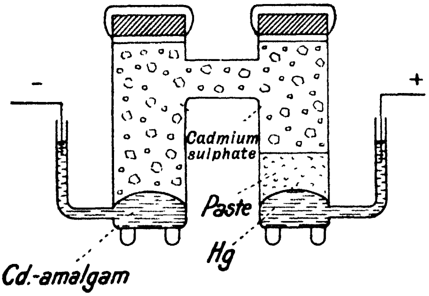

Fig. 9

Additional Apparatus

Normal Cadmium Cell.—Fig. 9 shows the construction of the cell. The current is produced as a result of the reaction expressed in the equation :

$$\mathrm{Cd} + \mathrm{Hg_2SO_4(solid)} = \mathrm{CdSO_4(solid)} + 2\mathrm{Hg}.$$

Prepare the cell for use thus. Clean the glass vessel with hot nitric acid, wash it with distilled water, pass steam through for 15 minutes, wash again with conductivity water and dry. Warm one part of Kahlbaum's purest cadmium with seven parts of purest distilled mercury and pour the amalgam produced into one leg of the vessel until a layer about 0·5 cm. thick has been formed. The amalgam is liquid at the temperature of the water-bath. It solidifies soon on cooling. Into the other leg pour distilled mercury to form a layer 0·5 cm. thick and cover with a paste made from solid Hg_2SO_4,[1] ground crystals of $3CdSO_4 . 8H_2O$ and Hg which are stirred to form a thick "porridge" with saturated $CdSO_4$-solution. The paste should be of sufficient thickness to support a layer of $CdSO_4$ crystals without appreciable immersion of the crystals. Into the space above the Cd-amalgam and into the tube which connects the two legs pour small crystals of $3CdSO_4 . 8H_2O$, fill up with a saturated solution of this salt which has been kept standing in contact with the solid and seal both legs by pouring in paraffin wax. Finally, close the legs with corks saturated with the wax, taking care that small air spaces remain under them: otherwise the glass will crack in hot summer weather. Protect the parts of the cork which project by covering them with sealing wax. Between 15° and 25° the E.M.F. of the normal cadmium cell amounts to

$$1{\cdot}0183 + 0{\cdot}00004\ (20° - t°)\ \text{volt.}$$

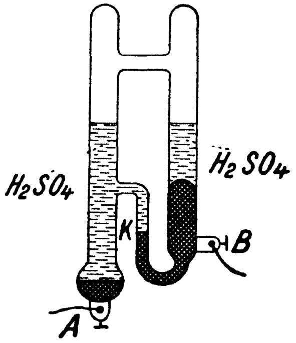

FIG. 10

The temperature coefficients may be neglected since the corrections involved lie within the limits of error of the method.

Capillary Electrometer.—The use of this device as a null instrument depends on the following considerations. The surface tension of mercury in contact with dilute sulphuric acid depends on the magnitude of the charge which the metal acquires with respect to the acid. Since there are always some Hg-ions in the H_2SO_4 (because of production of oxide) and since their osmotic pressure is greater than the solution pressure of Hg, the Hg is usually positively charged. Consequently, the Hg rises to a corresponding level in the capillary tube *K*. If now a negative current is passed from *B* to *A* (Fig. 10), positive Hg-ions will lose their charges at the mercury meniscus and the charge there will be reduced (" polarisation "), the surface tension will increase and the meniscus becomes flatter. But if the negative current passes from *A* to *B*, the number of Hg-ions at the surface of the meniscus increases, the electric charge is increased and the surface tension decreases. Accordingly, the curvature of the meniscus becomes more pronounced.

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[1] To be had from the firm of de Haën, Seelze bei Hannover.

The degree of polarisation must never become great and it must be counteracted again as rapidly as possible. Hence, until some measure of compensation (see next section) has been attained, the circuit is closed only for very short periods and during the rest of the time the instrument is short-circuited. A suitably constructed current breaker automatically ensures that these conditions are fulfilled (cf. Fig. 11).

MEASURING THE p_H

From what has been said above it follows that the determination of H-ion concentration is equivalent to the measurement of a potential difference. In the method which is by far the most commonly used advantage is taken of Poggendorf's *principle of compensation.*

Use of a Wire.—Connect the ends *b* and *d* of a uniform resistance wire, 100 cm. long, to an accumulator *A*, thus creating the potential difference E_A (larger circuit). By means of the sliding contact *c* particular potential differences E_1 can be established. These differences are easily calculated from the equations (cf. Fig. 11):

$$E_A : E_1 = 100 : bc\,; \quad E_1 = \frac{bc}{100} E_A.$$

If an opposing potential is established by means of a galvanic circuit *K* having E.M.F. = *E* the corresponding value for the section *bc* being E_1 (*e.g.* at *b* positive pole of the circuit opposed to positive pole of the accumulator) then, in general, a galvanometer or capillary electrometer in this " small circuit " will give a reading. But by moving the contact *c* E_1 can be made equal to *E* and the null point instrument remains at rest. The E.M.F. of the cell is then equal to the part of the potential of the accumulator calculated according to the above formula. Care should be taken that the sliding contact is adjusted as nearly in the middle as possible (cf. the following paragraph).

The voltage E_A of the accumulator must be determined each time before an actual measurement is made. This is done by comparison with the normal cadmium cell *NE* which is introduced into the small circuit. The sliding contact is then moved until no current passes. If a see-saw switch is used it is very simple to standardise the accumulator and, immediately after, merely by moving the see-saw into the other position, to proceed to the measurement in hand.

Further, as can be seen in the diagram, it is easy to step up the voltage, when necessary, in the case of especially low E.M.F. Such E.M.F. are present when the sliding contact has been set near *b*. In these cases the normal cell is opposed to the chain or in series with it but, naturally, account must be taken of this when the actual measurement is made. For convenience a switch should also be used. When the capillary electrometer is used as a null instrument it is useful to substitute an electrometer key for the switch. When the apparatus is not in use the former causes a short-circuit.

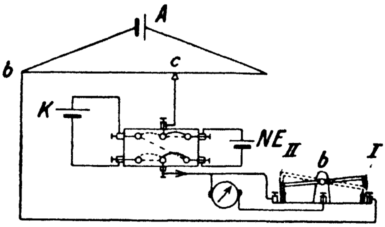

FIG. 11

⌒ = Position of the see-saw when standardising the accumulator.
⁀ = Position when measuring the E.M.F. of the chain.
---- = Position when measuring the E.M.F. of the chain when *NE* is in the circuit.
I = Position of the electrometer key when not in use.
II = Position of the electrometer key when a measurement is being made.

Measurement with the Rheostat Device of Michaelis.—Since the resistance of the wire used for measurement is rather small a rather considerable current is taken from the accumulator during the measurement. This leads to variations in the source of the current. Consequently, the wire is often replaced by resistance boxes. A particularly advantageous apparatus is that of Michaelis which permits the E.M.F. of a chain to be determined without calculation (Fig. 12).

Two rheostats with plugs are connected in series with a variable resistance *SW* of the sliding type, a switch and an accumulator *A* (large circuit). The small circuit, which consists of the normal cell or later the chain to be measured (in opposition to the accumulator), the capillary electrometer and a switch, is attached to rheostat I.

At the beginning of the determination both the sliding resistance *SW* and rheostat I are completely isolated (the rheostat by pushing all the plugs firmly in) but all the elements of the box II, on the other hand, are included in the circuit (all the plugs being removed). Then from rheostat I so many plugs are taken and inserted into the corresponding holes of rheostat II that in I (small circuit) the resistance amounts to 1018Ω. The fraction of the voltage of the accumulator which then passes through I is expressed by the formula:

$$\frac{R_{\text{I}}}{R+R_{\text{II}}}E_{\text{A}}.$$

The opposing potential difference of the normal cell—amounting to 1018 millivolts, and also passing through I—will not, in general, compensate this part of the current from the accumulator and, accordingly, when the switch is used the level will rise in the capillary electrometer. But the part of the current from the accumulator affecting I can be reduced to exactly 1018 millivolts by adjusting the sliding resistance, and then the electrometer remains unaffected. Consequently, each ohm of resistance offered by I corresponds to exactly 1 millivolt. By diminishing the resistance in I and correspondingly increasing it in II (moving the plug from II to I) the potential between *b* and *d* in the large circuit is not affected but, of course, the part of the voltage affecting I is reduced by a number of millivolts equal to the reduction in the number of ohms. Thus, when the normal cell is replaced by the chain to be examined, the resistance in I, after equivalence has been attained, gives directly the E.M.F. of the chain.

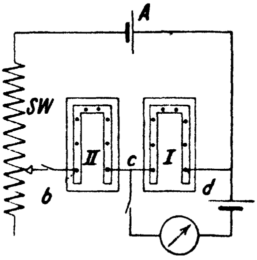

FIG. 12

Ionometer giving direct p_H Readings.—Various types of apparatus for the potentiometric determination of p_H are on the market. Those models which contain a measuring bridge, a sensitive galvanometer, batteries and so on, well protected in a portable case, combine the advantage of simplicity in use with great accuracy (0·01–0·02 p_H). Two shunts make possible measurement with the *saturated* calomel electrode or the quinhydrone electrode. Usually, such an apparatus gives direct p_H readings, a diagram for temperature correction being provided.

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2. GENERAL METHODS OF PURIFICATION

Most substances which exhibit potent physiological action, such as enzymes, hormones, vitamins and so on, occur, as has already been said, in minute traces only in nature. To-day the efforts of physiological chemists are directed in a very special degree to the investigation of these substances. In order, therefore, that they may be detected and isolated they must first be freed from relatively large amounts of accompanying material. Thus, it was necessary when isolating the female sex hormone from urine to attain an approximately 54-thousandfold increase in purity, and in the case of the substance which regulates increase in length in plants (auxin-*a*), a 21-thousandfold increase was necessary.[1] A systematic procedure for such isolation can only be worked out if it is possible to follow quantitatively during the various stages of purification the amount and concentration of the substance sought. Chemical reactions of the substance itself capable of being used for quantitative assay, colour reactions for example ($FeCl_3$ reaction of adrenaline, $SbCl_3$ reaction of vitamin-*A*), are only very rarely at the disposal of the investigator. Generally, the amount and concentration of such a substance must be deduced from its biological action: in the case of enzymes, for example, from the velocity of enzymic transformations, in the case of hormones and vitamins from their specific actions on animals (examples: determination of the invertase of yeast by measuring the extent of hydrolysis of sucrose; determination of insulin by measuring the diminution of the sugar content of the blood; determination of the male sex hormone by means of the "cock's comb test"; determination of the antirachitic vitamin by observing the power to cure or prevent experimental rickets; determination of the plant growth-substance by measuring the increase in length or the curvature of seedlings, and so forth). All these methods of determination or testing must be carried out under exactly defined conditions so that comparable results may be obtained. So long as the active substance is not known in the pure state the results of these tests can be expressed in relative or arbitrarily chosen units only and not in absolute figures, the units corresponding to precise quantitatively defined activity. It follows at once that the yield at the various stages of purification is expressed by the number of units present at each stage. The number of units per unit of weight of the material obtained is taken as a measure of the degree of purity attained.

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[1] Cf. Kögl and co-workers, *Z. physiol. Chem.*, **1933, 214, 241.**

When active substances are not already present in *solution* in natural secretions or excretions (*e.g.* blood, urine and the like) they must first be extracted from the animal or vegetable material and brought into solution. According to the nature of the substances the solvents used may be water, salt solutions, dilute acids and alkalis or organic liquids. Enzymes and many hormones must be extracted in the cold under the mildest possible conditions. More resistant substances, on the other hand, are preferably extracted hot. Hot extraction is of particular importance when it is feared that the substance to be isolated may be destroyed by enzymes present in the starting material. (Example: hot extraction of glucosides from plant material.)

The bringing into solution of certain substances of physiological importance while completely preserving their activity and at the same time attaining the highest possible degree of purity is by no means always a simple operation. It involves "selective" separation from cells and tissues, the smallest possible amounts only of impurities being retained. The most comprehensive study of the process so far made was that of Willstätter in his investigations on the peroxidase of horse-radish, the enzymes of yeast and the enzymes of the digestive systems of animals. In the case of the peroxidase, for example, the concentration of the enzyme can be increased almost 200-fold, simply by choosing a suitable method of extraction.[1] In many cases the process is not one of simple, purely physical dissolution. For example, with many enzymes which are bound in the cells to certain insoluble materials, such as insoluble proteins or carbohydrates, it is rather a process of liberation. In order to convert such bound enzymes (" desmo-enzymes ") into soluble enzymes (" lyo-enzymes ") it is necessary to decompose chemically the substances responsible for the binding. This decomposition may sometimes be brought about by enzymes.

By whatever method the solution of the crude product is obtained, it naturally always contains complicated mixtures of substances of the most different kinds, some of low and some of high molecular weight. Simple procedures which can generally be applied for separating such mixtures into fractions of different particle size are known. They will now be described.

DIALYSIS AND ULTRA-FILTRATION

In both processes advantage is taken of the general principle that membranes having pores of appropriate size (animal membranes, *e.g.* bladders of fish ; parchment paper, collodion, cellophane) are impervious to substances of high molecular weight but permit those of low molecular weight to pass through. *Dialysis* is most simply carried out by hanging a tube or bladder made of some such material containing the mixture in a large vessel of water or in running water. In the course of a few days all the constituents of low molecular weight (such as salts, simple organic acids or bases, sugars, amino-acids, purines and so forth) pass through the membrane into the surrounding medium, whilst proteins, carbohydrates of high molecular weight, enzymes, many hormones and toxins and so on, are retained. Suitable antiseptics, such as toluene, chloroform, phenols and the like, should be added to the internal and external liquids to prevent bacterial decomposition.

In *ultra-filtration* suction is applied to some suitable membrane. The colloidal constituents of the mixture and the constituents of high molecular weight remain in the filter. The degree of permeability can be varied, within certain limits, in both processes by a suitable choice of material for the membrane. Before use membranes must be carefully tested for the presence of holes. This is best done with dyes in colloidal solution such, for example, as litmus. *Collodion membranes may be prepared* in the following way.[1] The advantage of this process is that the troublesome removal of the membranes from the walls of the mould is simplified.

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[1] Willstätter *et al.*, *Annalen*, 1917, **416**, 21 ; 1920, **422**, 47.

Dip suitable moulds made of glass, *e.g.* test-tubes, into a bath of caramel at 200° and withdraw them, at the same time rotating them so that they become covered with a uniform layer of caramel. Lay them aside for about 1–2 hours until the layer has cooled and solidified and then dip them, at the same time rotating them, in collodion solution. (The diameter of the pores depends on the nature of the collodion solution ; a 6 per cent alcohol-ether solution containing 3 per cent of *n*-butyl alcohol is recommended.) Now set the moulds in a vertical position and dry for 6 hours at 37°. Then after 6 hours dip again in the solution and dry as before. Finally, repeat the dipping and drying a third time in the same way and place the moulds in 30 per cent alcohol at 80°. The layer of caramel soon dissolves and collodion tubes slip off undamaged of their own accord. Remove sugar from them by changing the alcohol after some time or simply by washing in running water. If kept in 70 per cent alcohol covered with a layer of toluene such collodion tubes can be kept in a sterile condition, without change of pore diameter or power to resist pressure, for an indefinite period.

In suitable cases dialysis may be carried out considerably more rapidly than when the ordinary procedure is applied by using the so-called *rapid dialysers*. These are devices having membranes of considerably larger surface area and appropriate stirrers which keep the liquid in rapid motion both inside and outside the membrane.

Electrolytes are much more thoroughly and rapidly removed if dialysis in an electric field, the so-called *electro-dialysis*, is substituted for ordinary dialysis. The simple process of diffusion is then reinforced by the electrical transfer of the ions. Fig. 13[2] shows a common type of electro-dialyser. In recent years, devices which combine the great advantages of the principles of rapid and electro-dialysis have also been introduced.[1] When sensitive substances are being purified by electro-dialysis it has to be borne in mind (1) that when the current density is of considerable magnitude the material may occasionally become rather *warm*; (2) that as a result of ionic migrations the *reaction may be considerably displaced* to the acid or alkaline side; (3) that such displacements are connected with the *precipitations* which frequently occur during electro-dialysis. Generally, such precipitation results from the flocculation of colloids which are stable only in presence of dissolved electrolytes. Occasionally, such precipitates carry down considerable amounts of active material, *e.g.* of enzymes.

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[1] *Biochem. Z.*, 1933, 266, 128.

[2] Other designs are described in Rona, *Praktikum der physiologischen Chemie*, I. *Fermentmethoden* (Berlin, 1931).

Active substances of low molecular weight are concentrated and purified in principle according to the procedures commonly applied in organic chemistry, namely, separation with suitable solvents, production of difficultly soluble salts or other derivatives, distillation, crystallisation and so on. *Substances of very high molecular weight* are never isolated by distillation and only very rarely by crystallisation. They are rather purified by *adsorption methods* which can be modified in many ways and are of very general application.

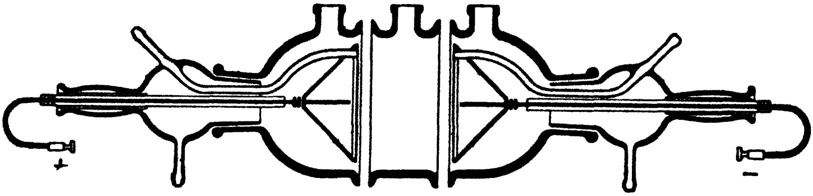

FIG. 13

PURIFICATION BY ADSORPTION

Purification by adsorption depends essentially on the fact that almost all substances of high molecular weight—and to a smaller extent many simpler compounds also—are tenaciously retained by powerful forces acting at the surface of materials in which the surface activity is highly developed. Such materials are, for example, alumina, colloidal hydrated oxides of iron, kaolin, silicic acid, fuller's earth, animal charcoal and also organic compounds, such as tristearin, cholesterol, fibrin, casein and so on. Since the relation between the nature of the adsorbing surface (the adsorbent) and the material to be adsorbed from the solution (the adsorbate) may be very specific and greatly influenced by the conditions of the system (*e.g.* the concentration of $H^{\cdot}$-, OH'- and other ions, the degree of dilution and so forth), the various constituents of the mixture being often affected in very different degrees, it follows that, by suitable choice of adsorbent and of conditions, the various substances of high molecular weight can be separated from each other and particular substances extracted from complicated mixtures. In order to recover the adsorbed material, the adsorbent with the adsorbate is separated by filtration or better by centrifugation and " eluted " with a solution, the composition of which favours the dissolution of the adsorbed material. In most cases a simple change in the hydrogen-ion concentration suffices; thus very many substances can be adsorbed from faintly acid media and can then be eluted with alkaline liquids which frequently should contain polyvalent ions (*e.g.* those of phosphates) which either react chemically with the adsorbent or are themselves adsorbed and so tend to drive out the already adsorbed material. In other cases the adsorption is carried out from aqueous-alcoholic solution and the elution with pure water. When salt solutions (*e.g.* secondary phosphate or bicarbonate) are used for elution it is frequently necessary to remove the electrolytes afterwards by dialysis.

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[1] Brintzinger, *Kolloid-Z.*, 1933, **66**, 183.

The use of adsorbents having their chemical and physical properties defined as accurately as possible is of special importance for the success of the adsorption. The work of Willstätter and his school has shown, in the case of the alumina gels most frequently used, that when they are prepared in the appropriate manner they exhibit the compositions of accurately defined hydrates of aluminium oxide. Thus, several species of aluminium *ortho*hydroxide having the formula $Al(OH)_3$ (alumina C_α, C_β and C_γ), aluminium *meta*hydroxide having the formula AlOOH, and the di-, tri- and polyhydroxides (alumina A and B ; Bayerite) obtained from one or more molecules of the *ortho*hydroxide by elimination of water, are distinguished.[1] These compounds were subjected to analysis after the adsorbed moisture had been removed, under the mildest possible conditions, with organic solvents (acetone, then petrol ether).

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[1] Cf. in this connexion Kraut, *Kolloid-Z.*, 1929, 49, 352; *Ber.*, 1931, 64 [B], 1697.

Similar considerations apply to the various hydrated oxides of iron and of silicic acid.

Recently, also, well-defined oxides or hydrated oxides of iron, aluminium and other elements which occur in nature as homogeneous crystalline compounds (*e.g.* bauxite, haematite, diaspore) have been used with success as adsorbents. These crystalline compounds usually have low adsorbent power, but, on the other hand, are very specific and are easily obtained.

PREPARATION OF THE MOST IMPORTANT ALUMINA GELS

Alumina B.—Dissolve 250 g. of $Al_2(SO_4)_3.18H_2O$ in 750 c.c. of water, warm to 48° and pour the solution rapidly with very vigorous mechanical stirring into 2500 c.c. of ammonia solution (15 per cent by weight) which has been warmed to 48°. The temperature rises to 50°. Continue stirring for 30 minutes, maintaining the temperature at 48°–50°. The very voluminous precipitate becomes somewhat thinner during this period but just not flocculent. Dilute the suspension to 12 litres in a filter jar and wash as thoroughly as possible by frequent decantation with water. Before the fourth decantation stir the precipitate with an equal volume of 15 per cent ammonia solution, in order to decompose the traces of basic sulphate still present, and after 5 minutes make up to 12 litres with water. On the day of precipitation wash by decantation at least once more (*i.e.* five times in all) so that the alumina does not remain over night in contact with concentrated ammonia solution. The next day continue the washing until, on three successive occasions, the water does not remain clear (12 to 14 times altogether). During the last washings the precipitate will become continually more compact so that at the end the wash-liquor can be poured off almost completely from the rather plastic gel.

Alumina C_α.—In the preparation of this form use preferably ammonium alum rather than ordinary aluminium sulphate, on account of the constant water content and greater purity of the former. On each occasion measure the ammonia solution accurately and determine its ammonia content by titration. Hence, weigh out accurately the corresponding amount of alum.

Pour 100 c.c. of 10 per cent ammonia solution into 600 c.c. of water at 63° containing 22 g. of ammonium sulphate and bring the temperature quickly to 58°. Stir vigorously with a mechanical stirrer and pour in rapidly, in one lot, a solution of 76·7 g. of ammonium alum which is at a temperature of 58°. The temperature rises to 61°. Do not let the temperature fall below 58°, and after 10 minutes separate the precipitate from the mother liquor in a rapidly moving centrifuge. Wash five times on the centrifuge, each time transferring the gel to a flask and shaking with 1500 c.c. of water. To the first wash-water add 1·25 g. of ammonia and to the second twice as much before washing. On centrifuging for the sixth time the supernatant liquid remains turbid. The precipitate then contains only traces of sulphate. Carry out the whole operation rapidly because the α-modification is not stable. A little more than 2 hours should suffice between the beginning of the precipitation and the completion of the washing.

Alumina C_β.—A few hours after it has been precipitated the α-compound changes into the β-compound and the appearance of the gel also changes. The flocculent suspension becomes a compact plastic mass with a yellow tinge. The change can be detected qualitatively by means of a solubility test with acid.[1] The β-form is the less soluble.

Alumina C_γ.—Precipitate as described for C_α but wash by decantation, and do not centrifuge. Stir for 15 minutes at about 60° and then transfer the mixture to a filter jar. Wash each time with 5 litres of water. (Larger amounts of material can be handled without difficulty, but, of course, the amount of wash-water must be correspondingly increased.) The precipitate settles rapidly at first, leaving the supernatant liquid clear, but forms a rather tall column. At the fourth washing add to the wash-water, before use, 80 c.c. of 20 per cent ammonia solution, in order to decompose basic aluminium sulphate. Continue to wash repeatedly (from 12 to 20 times) until the wash liquor no longer becomes clear. Then wash twice more by decantation. A few days at least are required for these two washings.

The alumina gel suspended in water usually requires some months at least for complete conversion into the γ-modification. The completion of the change can be recognised by the flocculent, non-jelly-like appearance, the diminished solubility in acid and alkali, and the behaviour towards hot ammonia.[1]

Alumina D.—Dissolve 130 g. of pure commercial aluminium hydroxide in a hot solution of 140 g. of 80 per cent potassium hydroxide in 900 c.c. of water, dilute to 1000 c.c., filter and dilute the filtrate to 10 litres. During 2 days pass into the solution a gentle stream of carbon dioxide, thus producing a granular precipitate. Decant the mother liquor from the thin layer of precipitate and wash twelve times with water containing carbonic acid. Then wash repeatedly with distilled water until the wash liquor remains turbid.

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[1] Cf. in this connexion Willstätter, Kraut and Erbacher, *Ber.*, 1925, 58, 2448, and in particular 2454.

Aluminium *meta*-Hydroxide.—Until recently the only known forms of the second monohydroxide of aluminium, the *meta*-hydroxide, were the crystalline minerals diaspore and bauxite. The *meta*-hydroxide is produced from the *ortho*-hydroxide at a high temperature. If the γ-modification is heated in a current of dry air the composition remains constant over a remarkably wide range of temperature, namely, between 210° and 240°. The substance then contains 16·5–16·6 per cent of water, whilst the calculated percentage of water for $AlOOH$ is 17·6. Corresponding ranges of temperature in which the composition remains approximately the same are observed during dehydration of the α- and β-modifications. But AlO_2H of uniform composition cannot be obtained in this way. In order to avoid secondary reactions it is better to heat an aqueous suspension of one of the alumina gels *rapidly* to 250° with ammonia in a sealed tube. The change in the *ortho*-compound which otherwise occurs at 100° does not then take place and a greyish gel rather more plastic than flocculent is obtained. This gel when dried with acetone has a composition approximately rather closely to that of the *meta*-compound (17·5–18·5 per cent of water). The final composition is attained on heating for 8–9 hours at 250° and is not further altered by longer heating.

Kaolin.—Commercial kaolin is best purified with hot concentrated hydrochloric acid before being used as adsorbent. If this is done enzymes adsorbed by it are more stable and the yields on elution are higher.

Mix 500 g. of kaolin well with 1500 c.c. of pure hydrochloric acid (*d* 1·18) and warm at such a rate that boiling begins only after one day. Then boil vigorously on the second day. Separate the iron-containing solution from the kaolin by repeated decantation after diluting with water and then repeat three times the treatment with acid. Altogether the purification requires 14 days for its completion. Finally, wash the kaolin with cold water but only to such an extent that the wash-liquor exhibits almost no acidity, whilst a small sample of the kaolin, on the other hand, still reacts strongly acid when placed on litmus paper. In some cases it has been found advisable exactly to neutralise the adsorbent with ammonia after removing most of the hydrochloric acid.

Adsorption from *mixtures*, such as those which are usually encountered when working with natural products, depends on a peculiarity which the individual adsorbable components exhibit. These components compete for place on the adsorbing surface and tend to expel each other in accordance with their " adsorption affinity ". If small portions of an adsorbent are added one after the other to such a mixture, the first portions adsorb predominantly, the constituents having the greatest affinity for the adsorbent. When these have been to a large extent removed, the remaining substances can be adsorbed by succeeding portions of the adsorbent. Consequently, it may happen, for example, that a particular enzyme is for the most part precipitated by the very first portions of an adsorbent but the reverse process may also occur, the accompanying materials being first removed, the enzyme itself being adsorbed only when further portions of the adsorbent are added. In the first case, the smallest possible amounts of adsorbent should be used in order to attain the highest possible degree of purification. In the second, the bulk of the accompanying material is first removed by addition of an amount of adsorbent accurately determined by preliminary test, and the enzyme itself is then adsorbed on further portions of the adsorbent. In the same way, it is occasionally possible to separate two enzymes from each other by successive adsorptions under the same conditions if their affinities for the adsorbent differ sufficiently (fractional adsorption).

Sometimes substances which exhibit close resemblances in regard to their *behaviour towards adsorbents* can be separated from each other by *elution* under different conditions. Thus, from mixtures of maltase and invertase adsorbed on alumina, primary phosphate at 0° removes practically only the invertase, whilst the bulk of the maltase remains on the adsorbent, and so can be obtained free from invertase by subsequent elution with diammonium hydrogen phosphate (cf. p. 131).

The attempt has been made to relate the process of adsorption of enzymes solely to the electric charges on the enzyme and adsorbent. Electro-negative colloids would be adsorbed by electropositive adsorbents and *vice versa* (L. Michaelis). In general, however, the attempt has not been successful.[1] There is no doubt that, in addition to the electrochemical forces, the residual chemical affinities of the adsorbent and the adsorbed substance, play a specific part which, however, cannot yet be further explained. Moreover, the electrochemical charges of enzymes and of other adsorbed substances are by no means constant. According to the degree of purity, and to the presence of the greatest possible variety of other substances, the charge on the enzyme is altered. The extent of adsorption also depends, in large measure, on the degree of purity and on the accompanying materials. Thus the amount of substance adsorbed may either increase or decrease as the degree of purity increases. The invertase of yeast, for example, is scarcely adsorbed at all from crude solutions by kaolin but is adsorbed from purified solutions with extraordinary ease. The amylase of the pancreas behaves in the opposite way.

From what has been said above it follows that the adsorption method can be applied :

(1) to the *purification* of enzymes, hormones, toxins and so forth (cf. pp. 70, 123) ;

(2) to the *separation* of the constituents of mixtures of such substances (cf. pp. 110, 132).

Finally, adsorption is applied also in the precipitation, separation and purification of many chemically more simple substances of medium molecular weight, *e.g.* natural pigments, such as chlorophyll, carotenoids, flavins and so on. Recently the following method of carrying out the adsorption of such substances has become general. This procedure, which is based on the original investigations of the botanist Tswett, is known as :

ANALYSIS BY CHROMATOGRAPHIC ADSORPTION[2]

The mixture of substances is dissolved in a suitable solvent (light petroleum, benzene, carbon disulphide, less frequently water), slowly drawn through a tube containing the adsorbent and cautiously washed (" developed ") with the same or with another solvent. In this way the various constituents of the mixture, according to the ease or difficulty with which they are adsorbed, are trapped in various zones, usually quite sharply defined, the most readily adsorbed constituents being concentrated in the upper zones and those which are less readily adsorbed in the following zones in the order of decreasing affinity for the adsorbent. Figs. 14*a* and 14*b* show a suitable apparatus and Fig. 15 is a diagrammatic representation of the appearance of the developed chromatogram obtained on adsorbing the crude hydrolysed pigment of the coloured wax of paprika on calcium carbonate.[1] The various zones are separated from each other mechanically, if necessary (when narrow tubes are used), by cutting the glass tube and are then eluted with suitable solvents (*e.g.* methyl alcohol, ethyl alcohol, pyridine and so on) which, if required, are used hot.

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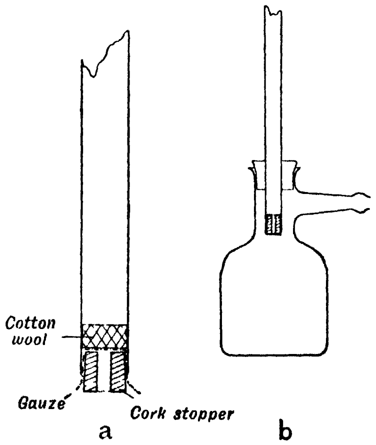
[1] *Biochem. Z.*, 1908, 7, 488 ; 1908, 10, 283 ; 1908, 12, 26.

[2] Cf. Willstaedt and With, *Z. physiol. Chem.*, 1938, 253, 40 ; Willstaedt, *L'Analyse chromatographique et ses applications* (Paris, 1938).



FIGS. 14*a* and 14*b*

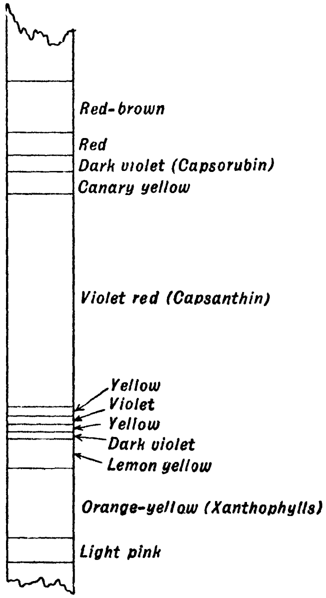


FIG. 15

In principle the method is also adapted to the separation of uncoloured compounds but the boundaries of the various zones cannot then be seen and the zones must be separated rather by appraisal and then analysed. With substances which fluoresce in ultra-violet light the analysis is very elegant. In this case the chromatogram is developed in a tube of transparent quartz and examined in the light of a mercury vapour lamp.

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[1] Zechmeister and von Cholnoky, *Annalen*, 1934, **509**, 276; *Die chromatographische Adsorptionsmethode* (Berlin, 1937); Cook, *J.S.C.I.*, 1936, **55**, 724.

The adsorbents commonly used are silicates, kaolin, fuller's earth; blood charcoal; a great variety of oxides including aluminium oxides, CaO, MgO, silicic acid (silica gel) and also, when organic solvents are employed, salts and various sugars. In the case of mixtures of substances having very different affinities for adsorbents it is preferable to use several adsorbents arranged in layers one above the other. As far as possible the adsorbents should be introduced into the tube in such a way as to form uniform layers and should be pressed down firmly. When large tubes are used it is advisable to suspend the adsorbent in an indifferent solvent and to draw the suspension through the tube by means of a water jet pump.

Procedure.—Moisten the whole column with a solvent sucked through at a pressure of about 60 mm. and as soon as the solvent has been almost completely drawn through allow the solution of the mixture to run in. Before the solution has completely soaked through, cautiously add the solvent to be used for " developing ". The more copious the washing the more will the various zones be separated from each other. Now draw off the solvent in an indifferent atmosphere, if necessary.

In general, the affinity for adsorption of chemically related substances decreases in the order: alcohols → ketones → esters → hydrocarbons. Usually, the capability to be adsorbed increases with the number of ·OH-groups and the number of double bonds in the molecule. Substances of high molecular weight are usually more easily adsorbed than are those of low molecular weight.

As a rule, it is not desirable to apply the adsorption method directly to very impure mixtures greatly lacking homogeneity, such as crude extracts of vegetable or animal products and the like, since under these conditions disproportionately large amounts of adsorbent are often used and in spite of that the degree of purification attained is rather inadequate. Accordingly, it is customary before purifying by adsorption to employ certain **preliminary methods of purification**. In the case of substances soluble in organic solvents the choice naturally falls, in the first place, on separation by means of suitable solvents. Substances which are soluble in water only (proteins, carbohydrates, purines, enzymes and so on) can usually be separated into various fractions by fractional precipitation with alcohol (possibly with alcohol plus ether and so forth).

Certain acids and salts of heavy metals which have been widely used as precipitants in physiological and chemical investigations are more specific in their action but for various reasons are not by any means always suitable for precipitating sensitive substances. Some of the more important of these precipitants are enumerated below.

Lead acetate (sugar of lead), precipitant for many proteins, tannins, nucleotides, purines, carbohydrate-phosphoric acid compounds and so on. Its action varies greatly in dependence on the p_H. The precipitates are decomposed with H_2S or H_2SO_4.

Mercury salts (acetate, sulphate, nitrate) precipitate proteins and certain amino-acids (tryptophan, cystine, cysteine, tyrosine), tannins, nucleotides and so forth. The precipitates are decomposed with H_2S.

Silver salts precipitate chiefly basic degradation products of the proteins (arginine, histidine), purines and many other bases. The precipitates are decomposed with H_2S and HCl.

Picric Acid, Picrolonic Acid, Flavianic Acid.—Precipitants for proteins, basic degradation products of proteins, purine bases, " biogenic amines ", alkaloids. The picrates, picrolonates and flavianates of these substances usually crystallise very readily. Some of them are easily, some sparingly, soluble. Picrates are decomposed with mineral acids, the liberated picric acid being extracted with ether, or are dissolved or suspended in absolute alcohol, into which hydrogen chloride is then passed. The organic bases then separate as hydrochlorides. Flavianates are decomposed with baryta (barium flavianate is sparingly soluble).

Phosphotungstic acid precipitates proteins and certain predominantly basic protein degradation products (arginine, histidine, lysine, cystine and, under certain conditions, proline and hydroxyproline also), many alkaloids and organic bases. The precipitates are decomposed with baryta or by treatment with sulphuric acid and extraction of the phosphotungstic acid with ether-amyl alcohol.

DRIED PRODUCTS. EVAPORATION TO DRYNESS

Yeast and bacterial cultures required for enzyme studies and the like can be obtained in dry form capable of being preserved for long periods simply by separating them from the nutrient medium, spreading them in thin films on filter papers, glass plates, porous plates or the like, and leaving them to dry at room temperature or at slightly increased temperature. Alternatively, they may be dehydrated with alcohol (or acetone) and then ether (or light petroleum). Animal organs also, after being ground up and dried, can be converted into stable powders in essentially the same way.

The simplest method of obtaining dry preparations of enzymes, for example, from aqueous solutions, is to precipitate, where possible, with alcohol, acetone or acetone plus ether (the solution is covered with one-third or one-half of its volume of ether and acetone or alcohol is added until the phases mix). Sensitive substances, however, may often be seriously damaged by this type of dehydration, enzymes being inactivated, and proteins denatured. In all cases it is important to work rapidly (centrifuge) at low temperature and frequently also to maintain a hydrogen-ion concentration which is especially favourable for stability, this concentration being not necessarily always that which corresponds to neutrality. In many cases it is preferable to evaporate the solution directly to dryness either in the Faust-Heim apparatus (a moderately warm air current being blown over the surface), in the evaporator of Gäde-Straub or, finally (in the case of small amounts of substance), simply in shallow dishes in a vacuum desiccator.

When *concentrating* solutions of enzymes and the like it is usually sufficient to distil in the vacuum of a water jet pump, provided that care is taken to distil rapidly and at the lowest possible temperature. It is preferable, however, to use one of the well-known makes of laboratory oil pump which must, of course, be protected from water vapour by means of suitable absorption apparatus between the pump and the distillation apparatus. In any case, more important than the efficiency of the pump is the use of a wide connecting tube to the condenser, the tube being as short as possible (when evaporating several litres of liquid the tube should be at no point less than 1·5–2·0 cm. in diameter) and of condensers of high efficiency (rapid condensers made of copper, cooling preferably with well-cooled liquids or freezing mixtures). Octyl alcohol should be added to prevent frothing.

CENTRIFUGING. CLARIFYING

In work with natural products, colloidal precipitates which can be filtered only with difficulty are encountered much more frequently than in the simple separations of inorganic and organic chemistry.

On the other hand, in carrying out all operations with sensitive substances the greatest possible speed is absolutely decisive for success. Accordingly, in biochemical work the *centrifuge* is used in most cases in place of the usual ordinary and suction filtering apparatus. Ordinary centrifuges are distinguished from centrifugal separators of the type used for milk, which are continuous in action.

Ordinary laboratory centrifuges of good quality can now be had from various makers. The largest of the usual models holds about 3 litres, usually in 6 tubes: the number of rotations per minute is 3000–6000. For the experiments recommended in this book a centrifuge capable of holding 100–200 c.c. will usually suffice. In laboratories in which series of large-scale biochemical investigations are carried out, three centrifuges (capacity 80 c.c., 300 c.c., 3000 c.c.) should be available, if possible. It is recommended that, in the smallest centrifuges, conical tubes, which greatly simplify the collection and washing of small amounts of precipitate, should be used. In the small and medium-sized centrifuges the tubes should invariably be of glass, and, in order to prevent breakage during the spinning, the metal holders for the tubes should be provided with rubber plates or rings laid on the bottom of the holders and the spaces between tubes and holders should be filled with water, or, still better, with glycerol. As a rule, metallic containers must be used in the largest centrifuges since even good quality glass is not sufficiently resistant. Suitable metals are: special steel, aluminium (hardest quality) and copper (toxic to enzymes!).

Where at all possible large centrifuges should be kept in a special room and regularly examined. The load should always be exactly equally distributed, the spinning should be started up slowly and attention should be paid to the lubrication.

Centrifugal separators are not at all expensive in view of the work which they perform. The sizes used in laboratories deal easily with from 30 to 40 litres of liquid per hour.

The recently introduced *Sharples centrifuge* which is much used in American laboratories is of the separator type. It rotates from 20 to 30 thousand times per minute and so precipitates all kinds of bacteria from suspension thus making it possible to produce sterile materials.[1]

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[1] The power W of a centrifuge is calculated from the equation $W = m.v^2/r$ where v is the radial velocity, r is the radius and m is the mass. Thus when the diameter is 30 cm. and the number of rotations per minute is 5000 a force 4210 times that of the earth's gravitational field is developed.

The ultra-centrifuge devised by Svedberg is still more powerful. It has been used for the determination of the molecular weights of proteins and the like.[1]

Solutions which can be freed only with difficulty from small traces of colloidal material are best clarified completely by filtration through *thin* (not exceeding 1 mm. thick) layers of kieselguhr. It is best to shake up the required amount of kieselguhr with some of the solution to be filtered, to pour the mixture on to the funnel and then to filter the rest of the solution. The filtering material sold by the Seitz firm in Kreuznach is very satisfactory for this purpose.

[1] *Nature*, 1937, 139, 1051.

II. BIOLOGICAL MATERIALS AND TRANSFORMATIONS

A. SUBSTRATES

1. PROTEINS

By far the greatest part of the organic material of the animal organism consists of proteins whilst in the vegetable kingdom the amount of carbohydrate greatly predominates. No living cell, however, is without protein. Proteins are characterised by their high molecular weight, and, chemically, by the fact that, on hydrolysis, they yield almost exclusively amino-acids frequently together with small amounts of carbohydrates (glucose, mannose, galactose, glucosamine), lipins, pigments, purines or phosphoric acids. Those proteins which contain constituents other than the amino-acid residues are usually called " compound proteins " in contrast to the class of " simple proteins ". But since recent investigations have shown that carbohydrate residues occur in most proteins, including the most important and especially in the albumins and globulins, this division is unsatisfactory. The most important groups of natural proteins will now be enumerated.

1. **Albumins.**—These are readily soluble in water at all reactions. On heating they are " denatured ", *i.e.* converted into forms insoluble in water but still soluble in alkalis and acids. They are easily isolated—from egg protein, milk, and serum, for example. If cautious methods for separating them are adopted they can be obtained in crystalline form. Saturation of their solutions with ammonium sulphate salts them out.

2. **Globulins.**—These are insoluble in pure water but soluble in dilute salt solutions, in acids and in alkalis. They are more easily salted out than are the albumins (by half-saturation with ammonium sulphate or saturation with common salt, for example). They are separated from albumins either by fractional precipitation (salting

out) or by dialysis, only the globulins being thus obtained. Closely related to the globulins are the myogen of muscle and fibrinogen, the coagulable constituent of blood.

A special position amongst the globulins is occupied by the vegetable globulins which are most easily obtained from grain seeds and seeds of the cucurbitaceae. They have been carefully studied[1] and found to crystallise readily.

3. **Vegetable Proteins soluble in Alcohol.**—Examples are hordein (from barley) and zein (from maize) and so on. In contrast to the proteins above mentioned, these are soluble in aqueous alcohol (*e.g.* 70–90 per cent), whilst they are insoluble in pure water and in absolute alcohol. They constitute the chief constituents of the gluten of grain seeds.

4. **Glutin.**—This is the chief constituent of the collagen fibres of the skin and tendons. On boiling it is converted into gelatin, but, if less drastic treatment is applied, slight degradation only occurs and glue is obtained.

5. **Scleroproteins.**—These are insoluble substances which are resistant to chemical and enzymic attack. Essentially they serve as supporting and enveloping material in the organism. They include:

(a) *Keratin,* the constituent of epidermis, hair, horns, hoofs, claws and nails. It is rich in cystine.

(b) *Elastin,* the fibrous constituent of ligaments, especially of those of the neck.

(c) *Fibroin,* the fibrous constituent of silk.

6. **Casein,** from the quantitative point of view the most important phosphorus-containing protein of milk. It separates from milk as a result of acidification or of treatment with the enzyme of rennet (rennin) in the presence of calcium salts, the calcium compound which is difficultly soluble being formed (preparation of cheese !). In the whey there remain albumin, globulins, lactose, salts and pigments including flavins. Once precipitated casein is insoluble in water but soluble in acids and alkalis.

7. **Mucins, Mucoids.**—These are the chief constituents of cartilage and of animal mucus. They are compounds of protein with chondroitin-sulphuric acid.

8. **Chromoproteins.**—The most important members of this

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[1] Cf. Osborne, *The Vegetable Proteins* (London, 1924).

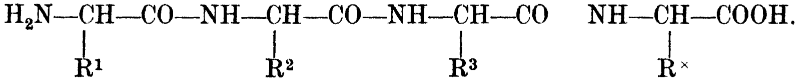
group are the blood-pigment (haemoglobin) and the respiratory pigment (cf. p. 197).

9. **Lipoproteins.**—A member of this group is the pigment of lobster shell. On heating or treating with alcohol it breaks down into a carotenoid dye (astacin) and a protein.[1] (Recall the red colour produced in the crab when it is boiled.)

10. **Nucleoproteins.**—These are the chief constituents of the nuclei of cells. They contain nucleic acid (cf. p. 80) usually combined with basic protein, *e.g.* histones and protamines.

11. **Histones and Protamines.**—These are proteins containing extraordinarily large amounts of diamino-acids (arginine, histidine, lysine). The protamines being of simple constitution and probably of relatively low molecular weight have been extensively studied.

The details of the chemical structure of the proteins have not yet been fully elucidated but it may be regarded as established that they consist of large molecules formed by the union of α-amino-acid residues. As in the synthetic polypeptides obtained by Emil Fischer [2] the residues are united by means of peptide linkages :



$$H_2N{-}\underset{\overset{|}{R^1}}{CH}{-}CO{-}NH{-}\underset{\overset{|}{R^2}}{CH}{-}CO{-}NH{-}\underset{\overset{|}{R^3}}{CH}{-}CO\quad NH{-}\underset{\overset{|}{R^x}}{CH}{-}COOH.$$

Like the amino-acids, the fundamental units of which they are composed, the proteins behave as " amphoteric electrolytes " towards both acids and bases. The p_H zone in which neither the anionic nor the cationic form but the electrically neutral, or, more correctly, the zwitter-ion occurs is termed the *iso-electric point.* At this point the maximal amount of flocculation occurs, the extent of swelling is minimal, the viscosity is lowest and so on. The iso-electric points of some proteins are, for example :

Ovalbumin	at p_H	4·8
Casein	„ „	4·8
Haemoglobin	„ „	6·8
Histone	„ „	8·5.

Those who wish to study the most important amino-acids obtained from proteins should consult appropriate text-books.

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[1] Kuhn and Lederer, *Ber.*, 1933, 66 [B] 488.

[2] Cf. Emil Fischer, *Untersuchungen über Aminosäuren, Polypeptide und Proteine*, vol. 1 (Berlin, 1906) ; vol. 2 (Berlin, 1923).

QUALITATIVE PROTEIN REACTIONS. DETECTION AND DETERMINATION OF SOME PROTEIN DEGRADATION PRODUCTS

Experiment.—Use for the following tests an approximately 1 per cent solution of ovalbumin,[1] an approximately 1 per cent solution of commercial peptone (Witte's or Merck's) and solutions of individual amino-acids, say, those obtained by complete hydrolysis of a protein (albumin, gelatin, casein).[2] Carry out the following tests comparing the various solutions with each other in regard to their behaviour on testing.

I. PRECIPITATION REACTIONS

(*a*) **Coagulation Test.**—Make slightly acid (the p_H attained should be about 5) with one drop of very dilute acetic acid and boil.

The coagulation test is pre-eminently a test for albumins. It is not sufficiently delicate for detecting traces of protein. For this purpose the following test, the so-called Heller ring test, is better.

(*b*) **Heller's Ring Test.**—Produce a layer of concentrated nitric acid under the solution. Any protein present is precipitated at the interface forming a turbid ring. Still more sensitive in some cases is the test with the substances mentioned under (*c*).

(*c*) Sulphosalicylic acid (10 per cent solution), *meta*-phosphoric acid, trichloro-acetic acid (10 per cent), tannin and acetic acid (Almen's solution, *i.e.* 4 g. of tannic acid in 8 c.c. of 25 per cent acetic acid + 190 c.c. of 40 to 50 per cent alcohol), phosphotungstic or phosphomolybdic acid (15 per cent solution, usually employed in solution in 5 per cent sulphuric acid), picric acid,[3] potassium ferrocyanide + acetic acid, potassium mercuric iodide and potassium bismuth iodide in hydrochloric acid solution.

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[1] The common commercial preparations usually contain varying amounts of insoluble denatured protein which must be removed by filtration. The student himself can prepare fairly pure solutions of ovalbumin from white of egg by diluting five-fold with water, so precipitating the globulins, filtering and concentrating the clear filtrate in a good vacuum. For the preparation of crystalline ovalbumin see Sørensen, *Biochem. Z.*, 1934, 269, 271.

[2] Boil 1–3 g. of protein for 24–40 hours under reflux with 20 parts of 20 per cent hydrochloric acid, dilute the solution, decolorise it as much as possible with animal charcoal, remove as much of the acid excess as possible by repeated distillation in a vacuum and neutralise.

[3] An aqueous solution containing 2 per cent of citric acid and 1 per cent of picric acid is known as Esbach's reagent and is used in medicine for the determination of protein in urine. The determination is simple. The precipitate, produced in a graduated cylinder, is allowed to settle for 24 hours and its volume is read off directly, or, better, the precipitated material is collected by means of a centrifuge.

(*d*) **Salts of Heavy Metals.**—Copper sulphate, ferric chloride, mercuric chloride, neutral and basic lead acetate, zinc sulphate, for example, likewise produce precipitates in many protein solutions. Some of these precipitates, however, are easily re-dissolved by an excess of the precipitant.

(*e*) **Salting Out.**—Add to a few cubic centimetres of a protein solution an equal volume of saturated ammonium sulphate solution. A precipitate indicates the presence of globulins. Now saturate the mixture by adding finely powdered ammonium sulphate. Albumin is precipitated. Thus are albumins separated from globulins.

II. COLOUR REACTIONS

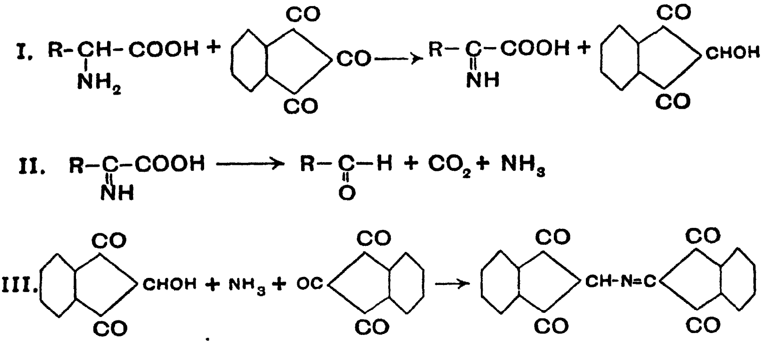
The first two of the following colour reactions are general reactions of proteins, the others are produced by individual amino-acids and are positive only when these are present.

(*a*) **Biuret Reaction.**—This is a general reaction of all proteins and of most of their degradation products. In order that it may be positive, at least two of the following groups must be present: $-CO{\cdot}NH_2$, $-CS{\cdot}NH_2$, $-C{:}NH.NH_2$, or $-CH_2.NH_2$, and must be united to each other either directly or by way of a N-atom or a third C-atom. The conditions are fulfilled by proteins, peptones, peptides and some other compounds, *e.g.* oxamide and amino-acetamide. Nevertheless the reaction is negative with some of the lower peptides and peptones.

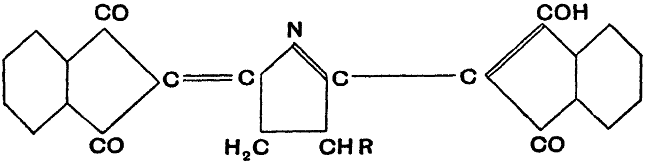
Apply the biuret test thus: add to the solution an excess of concentrated alkali and observe the colour which appears at ordinary temperature when a few drops of *very dilute* copper sulphate solution are poured in. The colour is red or reddish-violet.

(*b*) **Ninhydrin Reaction.**—Neutralise accurately an aqueous protein solution (which must be free from ammonium salts) and heat with a 1 per cent aqueous solution of triketohydrindene hydrate ("ninhydrin"). A beautiful blue colour, characteristic of all amino-acids, appears. This test is very delicate.

Probably the ninhydrin reaction of α-amino-acids is similar to the murexide reaction. The triketohydrindene dehydrogenates the amino-acid to imino-acid, being itself converted into the corresponding alcohol. The imino-acid decomposes into the next lower aldehyde, carbon dioxide and ammonia which condenses with the ninhydrin still present and the alcohol yielding the dye.[1]


I. R-CH-COOH + NH₂ CO CO CO → R-C-COOH + NH CO CHOH CO
II. R-C-COOH NH → R-C-H O + CO₂ + NH₃
III. CO CHOH CO + NH₃ + OC CO CO → CO CH-N=C CO CO CO

Proline and hydroxyproline react in a different way : one mole of the imino-acid and two moles of ninhydrin combine with elimination of water and CO_2 yielding a dye having the following formula [2] (Grassmann).



R=H *(proline)*
R=OH *(hydroxyproline)*

Both imino-acids condense in the same way with isatin. The best solvent for this condensation is glacial acetic acid.

(*c*) **Millon's Reaction.**—To prepare Millon's reagent dissolve mercury in twice its weight of nitric acid (*d* 1·42), at first in the cold then with heating, dilute with two volumes of water, allow to stand for some hours and pour off the clear liquid from the precipitate. When a protein solution is heated with a few drops of the reagent the precipitate produced acquires a red colour, or, if no precipitate appears, the solution becomes red. The reaction depends on the presence of tyrosine. Tryptophan gives a similar but yellowish colour.

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[1] Ruhemann, *J.C.S.*, 1910, 97, 1438, 2025 ; 1911, 99, 792, 1486.
[2] Grassmann and von Arnim, *Annalen*, 1934, 509, 288 ; 1935, 519, 192.

For the colorimetric determination of tyrosine in biological material compare the modification of Folin and Marenzi.[1]

(*d*) **The Xanthoproteic Reaction.**—This depends on the yellow colour given by most proteins with concentrated nitric acid. In this reaction the aromatic nuclei of tryptophan and tyrosine are nitrated. When sodium or ammonium hydroxide solution is added an intense brown colour is produced.

(*e*) **Pauly's Diazo-reaction.**—Make the protein solution alkaline with sodium carbonate and add a freshly prepared solution of diazotised sulphanilic acid.[2] A cherry-red colour, caused by coupling with the histidine and tyrosine present, appears.

Attempts have repeatedly been made to apply this reaction in the colorimetric determination of histidine and tyrosine.[3] The procedures adopted have been criticised by Kapeller-Adler.[4]

(*f*) **Knoop's Reaction.**—This reaction is specific for histidine. It is based on the fact that when histidine is warmed with bromine water there are produced, first a reddish to dark wine-red colour and then a flocculent black precipitate.

Recently the reaction has been applied in the colorimetric determination of histidine.[5] It happens that the flocculent black precipitate dissolves in ammonium carbonate solution producing an intense blue-violet colour the depth of which is proportional to the concentration of the histidine. In the determination of histidine in protein hydrolysates it is necessary first to isolate it from the mixture. This is best done with Hopkins' reagent (10 per cent solution of $HgSO_4$ in 5 per cent H_2SO_4) after the solution has been made neutral to litmus, sufficient 96 per cent alcohol has been added to produce a permanent turbidity, and a quantity of ether having one-third the volume of the alcohol used has been added. The precipitate which contains all the histidine is left overnight and then washed with alcohol and ether. Next the dry precipitate is dissolved in hot dilute hydrochloric acid, the solution is filtered and the mercury is removed from the filtrate by passing in hydrogen sulphide and filtering at the pump to remove the mercuric sulphide. After being neutralised the filtrate is concentrated, mixed with 10 per cent sulphuric acid and treated with 0·1 N-$KMnO_4$ solution, added drop by drop until a faint pink colour is produced. In this way the original colour of the solution is destroyed, and, after some time, a water-white liquid results. Bromine (1 per cent solution in 33 per cent acetic acid; 5 c.c. of bromine in 500 c.c. of glacial acetic acid made up to 1500 c.c. with water) is now added drop by drop until a very pale yellow colour, which persists for about 10 minutes, is obtained. Then 2 c.c. of a mixture of two parts of concentrated ammonia solution and one part of 10 per cent ammonium carbonate solution are added and the whole is heated for 5 minutes on the water-bath. A blue-violet colour appears. After some time the solution is diluted to some suitable concentration with ammonia-ammonium carbonate solution, or with 96 per cent alcohol, and the histidine is determined colorimetrically or with a step photometer. The mechanism of the reaction has not yet been completely elucidated.

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[1] *J. Biol. Chem.*, 1929, **83**, 89. Cf. also von Deseö, *Biochem. Z.*, 1934, **271**, 142.

[2] Diazotise 2 g. of sulphanilic acid in 3 c.c. of water and 2 c.c. of concentrated hydrochloric acid with a solution of 1 g. of potassium nitrite in 2 c.c. of water added in portions with cooling. Diazobenzenesulphonic acid is precipitated in crystalline form. Filter at the pump, wash, and, *while still moist*, dissolve in sodium carbonate solution.

[3] Kössler and Hanke, *J. Biol. Chem.*, 1919, **39**, 497.

[4] *Biochem. Z.*, 1933, **264**, 131.

[5] Kapeller-Adler, *Biochem. Z.*, 1933, **264**, 131; 1934, **271**, 206.

(*g*) The following are **reactions for the detection of tryptophan.** In the **reaction of Adamkiewicz** glacial acetic acid is added and then a layer of concentrated sulphuric acid is produced below the solution of the protein. According to Hopkins and Cole the reaction is due to the glyoxylic acid present in the glacial acetic acid. An intense blue-violet colour appears at the interface. The reaction is greatly affected by the copper content of the reagents. Extraordinarily small amounts of this metal considerably increase the intensity of the tryptophan reaction which may fail if they are absent.[1]

The **Voisenet reaction** is similar. It depends on the production of a blue-violet dye from tryptophan when a little formaldehyde is added and the mixture, in solution in concentrated hydrochloric acid, is oxidised with nitrite. The reaction has been applied to the *colorimetric determination of tryptophan.*

Procedure.[2]—If the protein of which the tryptophan content is to be determined is insoluble in water, heat 2 g. of it with 20 c.c. of 20 per cent of potassium hydroxide solution on the boiling water-bath until dissolution has taken place, filter and dilute to 50 c.c. Take 2 c.c. of the clear liquid and add to them one drop of 2·5 per cent formaldehyde solution. Then shake, add 15 c.c. of pure

[1] Winkler, *Z. physiol. Chem.*, **1934**, **228**, 50.

[2] Cf. Fürth and Dische, *Biochem. Z.*, **1924**, **146**, 275.

concentrated hydrochloric acid (*d* 1·175) and mix. After 10 minutes add, with shaking, 10 drops of 0·05 per cent sodium nitrite solution and dilute to 20 c.c. with concentrated hydrochloric acid. Make sure that the depth of colour is maximal by adding another drop of nitrite solution. If necessary, separate the liquid from precipitated potassium chloride and determine the tryptophan colorimetrically, or with a step photometer. For preparation of a standard solution for comparison use, preferably, a 5 per cent solution of sodium caseinate which contains 0·085 per cent of tryptophan. For the mechanism of the reaction see the paper by Kermack, Perkin, jr., and Robinson.[1]

Solutions of *free* tryptophan give a raspberry-red colour with bromine or chlorine water. The colour can be extracted with ethyl acetate or amyl alcohol. Excess of halogen destroys the dye with production of a yellow colour.

(*h*) **Lead Sulphide Reaction.**—Most proteins when boiled in concentrated alkaline solution with lead acetate produce a black or brown colour due to the liberation of H_2S from sulphur-containing amino-acids (cystine).

(*i*) **Nitroprusside Reaction.**—This reaction likewise depends on the presence of sulphur-containing constituents. A blue-red colour is produced with sodium nitroprusside in feebly alkaline solution. Since the reaction depends on the presence of the ·SH-group it occurs with cysteine, and, when KCN, which has a reducing action, is present, it occurs with cystine also.

(*k*) The **determination of cystine** by the method of Folin and Marenzi[2] depends on the blue colour which this amino-acid, after reduction with sulphite, gives with phosphotungstic acid.

Procedure.—Boil 5 g. of protein in a 300 c.c. flask with 20 c.c. of 6 N-H_2SO_4 and 2 c.c. of butyl alcohol for 18–20 hours. (Use a sand-bath and a flask with a ground joint.) Remove the butyl alcohol by distillation, dilute the liquid to 100 c.c., shake with 2 g. of kaolin for 5 minutes and filter through a folded filter paper. Take from 1 to 5 c.c. of the filtrate in a 100 c.c. measuring flask, add 2 c.c. of 20 per cent sodium sulphite solution and leave to stand for 1 minute. Then add 18 c.c. + x.0·5 c.c. (for each cubic centimetre of solution an additional 0·5 c.c.) of 20 per cent sodium carbonate solution, 2 c.c. of 20 per cent lithium sulphate solution, and, finally,

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[1] *J.C.S.*, 1921, 119, 1602.

[2] *J. Biol. Chem.*, 1929, 83, 103.

8 c.c. of uric acid reagent.[1] A deep blue colour develops immediately. Shake the solution, leave to stand for 3–5 minutes and make up to 100 c.c. with 3 per cent sulphite solution. As a standard for colorimetric comparison use a solution of cystine in N-H_2SO_4 containing 1 mg. of cystine per cubic centimetre. Treat this solution in the same way as the sample to be tested.

(*l*) **Detection and Determination of Hydroxyproline.**—Apply the procedures of Lang [2] and Waldschmidt-Leitz and his co-workers.[3] Convert the hydroxyproline into pyrrole by decarboxylation and oxidation, using hypochlorite, separate the pyrrole by distillation with steam and apply the sensitive colour test with dimethylaminobenzaldehyde or isatin and sulphuric acid.

Add to a few cubic centimetres of the solution of hydrolysed protein (containing about 0·1–3·0 mg. of hydroxyproline) 0·5 c.c. of 10 per cent sodium carbonate solution, 0·5–5·0 c.c. of hypochlorite solution [4] (0·5 c.c. for each 10 mg. of amino-acid mixture) and 1 c.c. of 10 per cent sodium glutamate solution. Pour the solution as rapidly as possible into an apparatus for steam distillation, wash in residual liquid with water and distil with steam until 100 c.c. of distillate are obtained.

For the reaction with *p*-dimethylaminobenzaldehyde take a portion of the distillate corresponding to 0·01–0·1 mg. of hydroxyproline, dilute to 20 c.c. with water, add 1 c.c. of a solution of *p*-dimethylaminobenzaldehyde and 1 c.c. of 6 N-HCl, allow to stand for 15 minutes at 20°–30° and then compare in a colorimeter with a freshly prepared standard solution of pyrrole (1 c.c. containing 0·01 mg. of pyrrole), which has been treated in the same way. For the reaction with isatin and sulphuric acid see Lang.[5]

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[1] Dissolve 100 g. of sodium tungstate in 200 c.c. of water in a 500 c.c. flask, add, with cooling, 20 c.c. of 85 per cent phosphoric acid, and pass in hydrogen sulphide for 20 minutes. After 4 minutes cautiously add 10 c.c. of phosphoric acid and filter from precipitated tungsten sulphide. Now shake the solution in a 1 litre separating funnel with 300 c.c. of alcohol. This causes separation into two phases. Run off the lower blue layer into a weighed 500 c.c. flask, make up to 300 g. with water and boil off H_2S. Then add 20 c.c. of phosphoric acid, boil under reflux for 1 hour, decolorise with a few drops of bromine, boil away the excess of bromine, and allow to cool. Further, dissolve 12 g. of lithium carbonate in a 500 c.c. flask, add first 25 c.c. of phosphoric acid and then, slowly, 150 c.c. of water, boil off the carbon dioxide and make sure that all the carbonate has dissolved. Cool, mix with the uric acid reagent and dilute to 1 litre.

[2] *Z. physiol. Chem.*, 1933, 219, 148. [3] *Z. physiol. Chem.*, 1934, 224, 187.

[4] Dissolve 80 g. of sodium hydroxide in 160 c.c. of water containing 60 g. of ice and pass in chlorine until the weight has increased by 71 g. Then make up to 1 litre with water.

[5] *Loc. cit.*, especially p. 151.

(*m*) **Detection and Gravimetric Determination of Arginine as Flavianate** (cf. Kossel and Gross [1]).—Remove barium from the solution of hydrolysed protein, make faintly acid with acetic acid, and, as far as possible, remove mineral salts. The concentration of the arginine should be about 10 mg. per cubic centimetre. Precipitate with a large excess of 5 per cent aqueous solution of flavianic acid. The beautifully crystalline yellow precipitate of arginine flavianate often comes down only after long standing and rubbing with a glass rod. Keep the mixture for two days in the refrigerator, decant the supernatant liquid through a weighed filter crucible, wash the precipitate in the vessel used for precipitation, add to the precipitate about 100 times its volume of very dilute flavianic acid solution and completely re-dissolve by heating on the boiling water-bath. Then leave to stand for 3 days. The now pure material separates completely in the form of very fine crystalline plates having a golden lustre. Filter through the crucible, wash with the smallest possible amount of water and dry to constant weight at 105°. One part by weight of the flavianate corresponds to 0·3566 parts by weight of arginine.

(*n*) **Proline and hydroxyproline** are relatively easily separated and detected in the form of their sparingly soluble reineckates.[2] A saturated solution of Reinecke's salt:

$$\left[\mathrm{Cr}\begin{matrix}(\mathrm{NH_4})_2\\(\mathrm{CNS})_4\end{matrix}\right]\mathrm{NH_4},$$

is added to the hydrolysed protein after its concentration has been brought to approximately 10 per cent and it has been made distinctly acid to Congo red with HCl. The two amino-acids separate in crystalline form. When mineral salts are present, precipitates, other than those of the proline and hydroxyproline salts, may be produced, thus giving rise to errors.[3]

DETERMINATION OF AMINO- AND CARBOXY-GROUPS IN PROTEINS, PEPTIDES AND AMINO-ACIDS; ANALYSIS OF PROTEINS BY DETERMINATION OF N-DISTRIBUTION ACCORDING TO VAN SLYKE

In the alkalimetric or acidimetric determination of amino- and carboxy-groups in amphoteric electrolytes, such as amino-acids, polypeptides and proteins in aqueous solution, serious difficulties are encountered, at least when ordinary indicators are used, because, in the titration of the carboxy-groups, the amino-groups also present interfere and conversely. The disturbing effect of the amino-group on the titration of the carboxy-group can be counteracted by titration in presence of formaldehyde (Sørensen's "formol" titration[1]); but, in general, the reciprocal effects of acid and basic groups on each other can be to a large extent prevented by carrying out the titration in a solvent having a dielectric constant smaller than that of water. The titration of the carboxy-groups of amino-acids and peptides in alcoholic solution by the method of Willstätter and Waldschmidt-Leitz,[2] using thymolphthalein as indicator, and the titration of amino-groups in aqueous acetone, using naphthyl red as indicator according to the procedure of K. Linderstrøm-Lang,[3] depend on this principle. For the theory of this method of titration see the paper by Linderstrøm-Lang.

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[1] *Z. physiol. Chem.*, 1924, **135**, 169.

[2] Spörer and Kapfhammer, *Z. physiol. Chem.*, 1929, **187**, 84.

[3] For the determination of proline with rhodanilic acid see Bergmann, *J. Biol. Chem.*, 1935, **110**, 471.

1. **Experiment. Determination of Amino-acids and Peptides by the Method of Willstätter and Waldschmidt-Leitz.**—Peptides of simple amino-acids can be quantitatively titrated with potassium hydroxide even in 50 per cent alcohol, using phenolphthalein or thymolphthalein as indicator. With free amino-acids, the concentration of the alcohol must be at least 90 per cent in order to counteract the disturbing influence of the amino-group and so to determine the carboxy-group. This difference can be used in forming an estimate of the proportions of amino-acid and peptide in a mixture of protein degradation products.

In order to determine the total carboxyl content of amino-acids and peptides dissolve about 0·3–1 millimole of substance in the minimal amount of water, add a few drops of an 0·5 per cent alcoholic solution of thymolphthalein, titrate with 90 per cent alcoholic $0{\cdot}2\,N$- or $0{\cdot}05\,N$-KOH until a blue colour develops, add absolute alcohol until the volume added is nine times that of the water originally taken for the dissolution thus causing the colour to disappear, and then complete the titration with the alcoholic solution of alkali. Carry out a blank experiment in which the amount of alkali consumed by a corresponding amount of 90 per cent alcohol is determined and is then subtracted from the value determined in the main experiment.

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[1] Sørensen and co-workers, *Biochem. Z.*, 1908, **7**, 45; *Z. physiol. Chem.*, 1910, **64**, 120.

[2] *Ber.*, 1921, **54** [B], 2988.

[3] *Z. physiol. Chem.*, 1927, **173**, 32.

When this procedure is applied to the titration of proteins, these are precipitated in small lumps when the alcohol is added so that difficulty is encountered. In this case it is recommended that the titration be carried out with not too-concentrated solutions and that heat be applied.

The practical application of this procedure to the measurement of degree of proteolysis is described below (p. 107).

2. **Experiment. Gasometric Determination of Free Amino-groups by Van Slyke's Method.**[1]—Free aliphatic amino-groups react with nitrous acid in the manner shown in the following equation :

$$RNH_2 + ONOH = ROH + N_2 + H_2O,$$

nitrogen being liberated. The volume of nitrogen is measured after the oxides of nitrogen produced at the same time have been absorbed in alkaline solution of permanganate.

The α-amino-groups of the amino-acids react quantitatively in this way in acetic acid solution within 5–10 minutes. The reaction with the ϵ-amino-group of free lysine proceeds more slowly and requires about 20 minutes. Amides, urea and the guanidine group of arginine in acetic acid solution, under the usual conditions, practically do not react to a measurable extent but react in solution in mineral acid. Hence, before the determination is made, mineral acids must be neutralised. The nitrogen of peptide linkages, as well as nitrogen in heterocyclic combination as in proline, hydroxyproline, tryptophan and histidine, do not react under the conditions of the Van Slyke method. With glycine, values which are too high are obtained, and this holds especially for peptides of glycine (error up to 20 per cent), which also invariably yield some carbon monoxide in the reaction.[2] Also, in presence of lower alcohols, values which are too high are obtained.

The following reagents are required for carrying out the determination :

(1) 30 per cent analytically pure sodium nitrite solution.
(2) Pure glacial acetic acid.
(3) Alkaline permanganate solution (50 g. of $KMnO_4$, 25 g. of KOH, 1 litre of water).

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[1] Van Slyke, *Ber.*, 1910, **43**, 3170; 1911, **44**, 1684.

[2] Abderhalden and Van Slyke, *Z. physiol. Chem.*, 1911, **74**, 505.

(4) Caprylic alcohol (secondary octyl alcohol). A few drops suffice to prevent frothing in protein solutions when shaken.

The reaction proceeds in a deamination vessel *C* (see Figs. 16*a* and 16*b*) having a capacity of about 50 c.c. This vessel connects with the tubes *A* and *B* by way of the stop-cocks *a* and *b*. One of the tubes (*A*) is used for running in the nitrite solution and the glacial acetic acid and the other (*B*, which may be graduated) is used for running in the liquid under examination. The amino-nitrogen content of the liquid should be sufficiently high to provide about 10–25 c.c. of nitrogen for measurement at the end of the determination. At the bottom of the deamination vessel there is another tube with stop-cock. This tube serves for running off the reaction mixture. The deamination vessel *C* has a constriction at the upper end and above the constriction a bulb which prevents the liquid in which the reaction occurs from spirting over into the attached capillary tube which is bent at a right angle. This capillary tube serves also for suspending the part of the apparatus which is shaken during the reaction and so is provided with a metal collar. By means of the three-way cock *c* the capillary tube can be opened to the atmosphere or to the gas burette as desired. The burette *D* and the capillary *k* are filled with water before each determination. The burette is firmly fixed but the deamination vessel and its attachments, which require to be shaken by means of a motor, are accordingly connected to the burette by means of glass to glass joints fitting as closely as possible and covered with soft, thick-walled rubber tubing. The stop-cocks on the apparatus should be held firmly in position with rubber bands and greased with a little viscous stop-cock fat of the best quality. The determination is carried out as follows:

Pour into the cylindrical tube *A* glacial acetic acid up to the level of the mark so that about one-fifth of the space (total capacity 45 c.c.) is filled. Run the acid now from *A* into the deamination vessel *C* and then fill *C* to the capillary tube with the nitrite solution. During these operations the three-way stop-cock *c* is in the position $\perp$ and the cock *d* on the burette is closed, whilst the connexion between *C* and *A* through the cock *a* is open. Shake the movable part of the apparatus, so causing production of a few cubic centimetres of gas which escapes into the atmosphere through the three-way cock *c* (now in position $\dashv$). Repeat this procedure once more so as to wash out the capillary with nitric oxide, continuing the

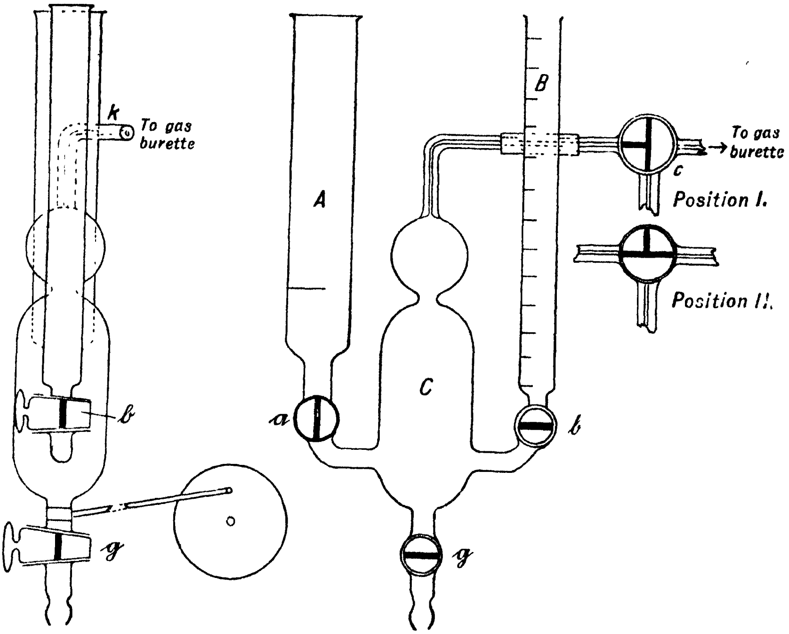

FIG. 16*a*

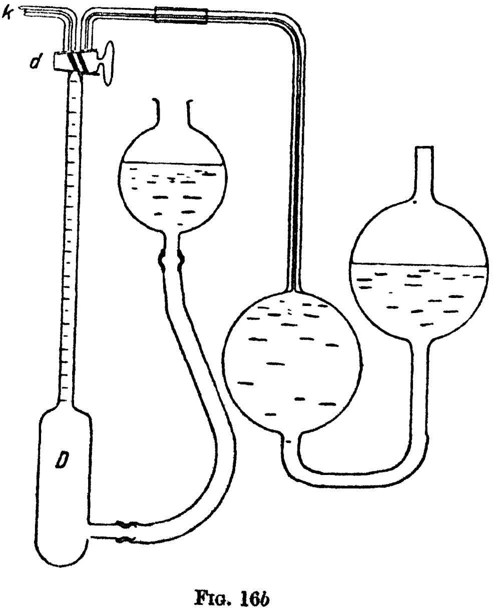

FIG. 16*b*

shaking until the volume of liquid in the deamination vessel is 20 c.c. [the cock *a* being open and the three-way cock *c* being closed (⊥)]. The required level for the liquid is usually marked on the vessel. Next close the cock *a* and, by turning the three-way cock *c* and the cock *d* (⑊), connect the reaction vessel with the burette. Now allow the solution to be analysed and some caprylic alcohol to run in from the tube *B* by opening the cock *b* (take care that no air is carried in with the solution) and wash residual solution in also with the smallest possible amount of water so that the deamination vessel is not more than two-thirds full. Close the cock *b* and shake at constant rate (300 revolutions per minute with an eccentric of 1·5 cm. displacement) for exactly 6 minutes. Then pour water in through *A* until the whole of the gas produced in the vessel *C* has been driven into the burette *D* and the liquid fills the whole of the capillary as far as the first boring of the cock *d*. Then immediately connect the burette with the absorption pipette containing the alkaline permanganate solution,[1] the cock *d* being in the position ⫽, and, by raising the levelling bulb, drive the gas into the permanganate solution. Shake the pipette for about 1 minute, drive the gas back into the burette and read the volume and repeat this procedure two or three times until the volume of the gas no longer changes. Calculate the weight of nitrogen obtained in milligrammes, taking into account the temperature and pressure and using tables in the usual way but divide by two in order to have the weight of amino-nitrogen, since half of the nitrogen produced comes from the nitrite. Expel the gas from the burette by way of the cock *d* (in position ⑊) and the three-way cock *c* (in position ⊢), allow the liquid to run off through the cock *g* and wash the deamination vessel several times with water. The apparatus is then ready for use in a new determination. When it is not in use the capillary tube between the burette and the Hempel gas pipette should not remain filled with permanganate solution but with water. The amount of nitrogen derived from the reagents should not exceed 0·3–0·4 c.c. of gas and should be determined by means of a blank experiment. It must then be deducted from the results obtained.

For the micro-modification of this procedure see Van Slyke's paper.[2]

3. **Experiment. Analysis of Protein by Van Slyke's Method.**—

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[1] 50 g. of $KMnO_4$ and 25 g. of KOH in 1 litre.

[2] *J. Biol. Chem.*, 1913, 16, 121; 1915, 23, 407.

In order that a protein may be accurately characterised the amino-acids which compose it and their amounts must be determined by the methods of Emil Fischer[1] or by that of Dakin,[2] for example (in any case the methods, described above, of determining the individual protein units must also be used). Somewhat simpler and in many cases sufficient is the separation into various characteristic groups of the mixture of amino-acids obtained by hydrolysis, employing the method of Van Slyke.

In Van Slyke's method of protein analysis advantage is taken of the fact that the mixed amino-acids obtained by hydrolysis of protein can be separated into two groups by treatment with phosphotungstic acid in solution in mineral acid. Arginine, lysine and histidine (together with part of the cystine) appear in the precipitate (" basic fraction ") produced by this reagent, whilst the remaining amino-acids appear in the filtrate (" mono-amino-acid fraction "). Of the *non*-precipitated amino-acids, proline and hydroxyproline are the only ones of which the nitrogen is not determined by the Van Slyke determination. Consequently, if one determines, in this fraction, the total nitrogen on the one hand (preferably by the micro-Kjeldahl method[3]) and the amino-nitrogen by Van Slyke's method on the other the difference represents the proline and hydroxyproline content, whilst the amino-nitrogen is derived from the remaining mono-amino-acids. In the basic fraction all the nitrogen of the lysine, one-third of the nitrogen of the histidine, and one-quarter of the nitrogen of the arginine react in the Van Slyke determination. Arginine can be determined either by alkaline hydrolysis' with liberation of ammonia or by precipitation as flavianate (by the method of Kossel and Gross—see above, p. 57). When it has been found how much of the total and how much of the amino-nitrogen of this fraction is derived from the arginine it is simple to calculate the way in which the remaining nitrogen is distributed between the lysine and the histidine. Further, the histidine can be directly determined colorimetrically (see above, p. 53).

The amide nitrogen which is eliminated during the acid hydrolysis of proteins is determined as ammonia before the various individual amino-acids are determined. And the " melanin nitrogen " which remains in the insoluble decomposition products after the hydrolysis must also be determined before the analysis and taken into account.

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[1] *Untersuchungen über Aminosäuren, Polypeptide und Proteine*, vol. i. (Berlin, 1906).

[2] *Biochem. J.*, 1908, 12, 290; *J. Biol. Chem.*, 1920, 44, 499; *Z. physiol. Chem.*, 1923, 130, 159.

[3] See in this connexion Pregl and Roth, *Quantitative Organic Microanalysis*, (London, 1937).

The analysis, which can be carried out with a relatively small amount of protein, is made as follows :

Boil 1–3 g. of protein under reflux with twenty parts of 20 per cent hydrochloric acid until the content of amino-nitrogen ceases to increase (as a rule about 40 hours are required). When hydrolysis is complete, concentrate in a vacuum until the liquid has the consistency of syrup and determine total and amino-nitrogen in a neutralised sample.

For determination of ammonia-nitrogen dissolve the syrup in water in a Claisen flask (capacity 1 litre) which is attached in the manner indicated in Fig. 17 to a distilling flask (capacity 1 litre) and to a smaller distilling flask (capacity 200 c.c.) which serve as receivers. The larger receiver contains 30–50 c.c. and the smaller 20 c.c. of $0{\cdot}1\,N$-H_2SO_4 together with a few drops of methyl orange solution. Now add to the liquid in the Claisen flask a slight excess of a 10 per cent suspension of calcium hydroxide, and, in order to prevent frothing, 100 c.c. of alcohol and distil for 30 minutes in a vacuum with a bath-temperature of 40°. Then combine the contents of the receivers and titrate the excess of sulphuric acid which they contain.

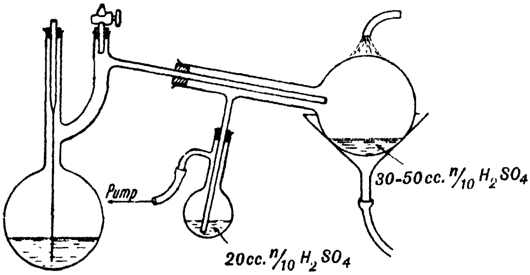
Pump
$30\text{-}50cc.\ ^{n}/_{10}\ H_2SO_4$
$20cc.\ ^{n}/_{10}\ H_2SO_4$

Fig. 17

The melanins present are found in the precipitate of calcium hydroxide. Separate this by filtration through a folded filter ; wash free from chloride and determine the nitrogen content.

Combine the washings and the filtrate from the calcium hydroxide precipitate, acidify with hydrochloric acid and concentrate to about 100 c.c. in a vacuum. Then add at once 18 c.c. of concentrated hydrochloric acid and 15 g. of phosphotungstic acid in 20 per cent solution. Without filtering make up to 200 c.c. and heat on the water-bath until the precipitated phosphotungstate has almost completely dissolved. Allow to stand for 48 hours. Then separate the granular crystalline precipitate by filtration with suction using a hardened paper on a funnel about 6 cm. in diameter and press down as dry as possible. Wash the not quite insoluble precipitate with 100–150 c.c. of a 2·5 per cent solution of phosphotungstic acid in 1 per cent hydrochloric acid. Cool this wash-liquor to 0° before use and apply it in six to eight portions, stirring thoroughly meanwhile with a glass rod bent to form a hook and flattened in the flame. Apply suction continuously and press down as dry as possible after each portion of wash liquor has been added.

In order to remove the phosphotungstic acid transfer the precipitate with 200–300 c.c. of water to a separating funnel (capacity 500 c.c.) add 10 c.c. of concentrated hydrochloric acid and shake with 100 c.c. of a 1 : 1 mixture of ether and amyl alcohol. Make sure that the solvents used contain no nitrogenous impurities. Repeat three times more the shaking with the ether-amyl alcohol mixture (using 50 c.c. on each occasion) and then shake the combined ether-amyl alcohol extracts with 50 c.c. of acidified water. Shake this water with 50 c.c. of fresh ether-amyl alcohol mixture and then add it to the solution of the hydrochlorides of the bases.

Remove phosphotungstic acid in a similar way from the filtrate obtained on precipitation with phosphotungstic acid. If it is desired simply to determine the ratio amino-N : total N in the filtrate, it is sufficient merely to neutralise the filtrate without previously removing the phosphotungstic acid.

2. CARBOHYDRATES

It is not proposed in this section to deal with the general chemistry and the constitution of the carbohydrates. Those who wish to acquaint themselves with the most recent developments in this field, developments which may be said to have reached a measure of completion, should consult the appropriate literature[1] and the modern text-books.[1] Although the aldehyde formulae for the simple sugars, as given by Emil Fischer, are still preferred by many workers, since these formulae seem to explain more clearly than do the new formulae many of the reactions (the behaviour towards oxidising agents for example), yet the student should familiarise himself with the new spatial formulae. Of these the following are recalled here:

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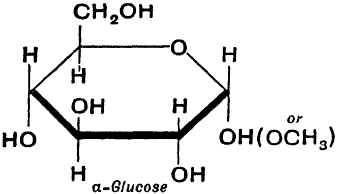
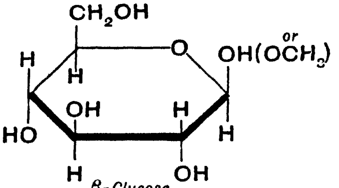
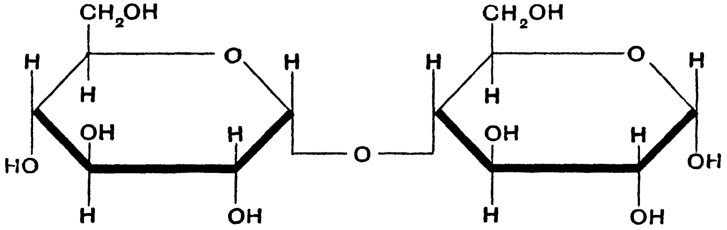
[1] Haworth and Hirst, *The Chemistry of the Carbohydrates and Glycosides* (*Ann. Rev. Biochem.*, 1937, 6, 99-114.)



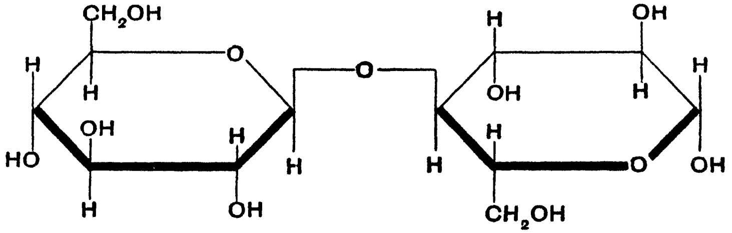
α-Glucose
(α-Methylglucoside)



β-Glucose
(β-Methylglucoside)



α-Maltose



β-Cellobiose

To the most important carbohydrates of high molecular weight such as starch and cellulose it is usual today to attribute chain formulae closely related to those used for maltose and cellobiose (see text-books).

The biological significance of the sugars, the part which they play in the energy exchanges in the organism (see p. 253) and the ways in which they are degraded biologically (see p. 233) are considered in subsequent sections of this book.

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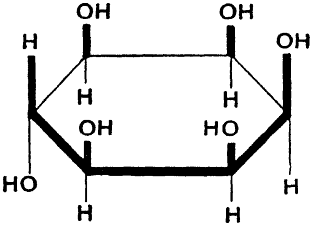
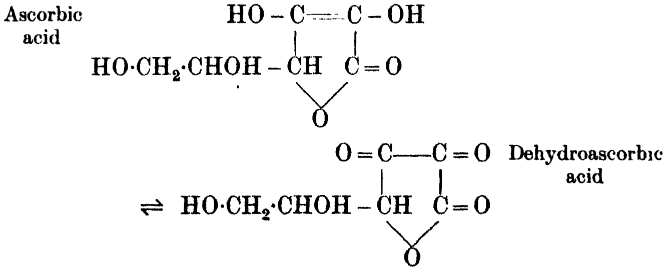
[1] Tollens-Elsner, *Kurzes Handbuch der Kohlenhydrate,* (Leipzig, 1935); Bernhauer, *Grundzüge der Chemie und Biochemie der Zuckerarten* (Berlin, 1933); Karrer, *Lehrbuch der organischen Chemie* (Leipzig, 1937).

In accord with the close inter-connexion which exists between all biochemical transformations it is found the carbohydrates also, like the sterols and bile acids, for example, can be converted into compounds of high physiological activity by certain routes which deviate from those normally followed during degradation. Attention may be drawn here to the relation of the carbohydrates to m-*inositol*, which, according to recent investigations by Eastcott,[1] appears to play an important rôle in the processes of cell division and may be regarded as a connecting link between the carbohydrates and the aromatic compounds. Most important of all, however, is the relation between the carbohydrates and the so-called *ascorbic acid*, *vitamin*–C the first vitamin to be obtained synthetically and to have its chemical constitution completely established. Ascorbic acid, like vitamin–B_2 (lactoflavin), which is one of the flavin pigments (see p. 70), and the sulphhydryl-compounds of the glutathione type (see p. 118), produces an oxidation-reduction system which may play a part in the regulation of the oxidation-reduction potential in the cells (cf. p. 179) and may possibly act catalytically in certain oxidation-reduction processes.

Inositol





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[1] *J. Physical Chem.*, 1928, 32, 1094; see also Kögl, *Ber.*, 1935, 68 [A], 16.

ISOLATION OF ASCORBIC ACID FROM PAPRIKA [1]

Mince 10 kg. of seeded paprika (*Capsicum annuum*) and add to the minced material a hot saturated solution of 1750 g. of barium acetate. Leave to stand for 1 hour and then press the pulp in a fruit press. About 8 litres of liquid are obtained and can be kept, if desired, for 2–3 weeks in absence of air. Decant the clear liquid, dissolve lead acetate in it so as to make the concentration of the latter 5 per cent and make slightly alkaline with ammonia (bromothymol blue colour just changed). Separate the precipitate in the centrifuge, suspend it in a little water and add 25 per cent sulphuric acid until the bromothymol blue acquires a faint red colour. Then remove lead sulphate in the centrifuge. Dissolve 10 per cent of barium acetate in the solution and again remove the precipitate with a centrifuge. Now dissolve again in the liquid a sufficient amount of lead acetate (5–15 per cent); at p_H 8·5 an excess of lead acetate should cause no further precipitation. Make alkaline to bromothymol blue, remove the precipitate in the centrifuge and repeat the treatment described above. The volume of liquid after the first treatment with lead is about 8000 c.c. and after the second about 1500–2000 c.c.

After adding ammonia (yellow colour with bromothymol blue), concentrate the liquid in a vacuum at 20°–30° to a syrup (volume 200–300 c.c.). With continuous stirring add two volumes of methyl alcohol and remove, by filtration with suction, the almost inactive precipitate produced. Then add one volume of acetone and decant from the resinous precipitate which is produced. Extract this precipitate with a little methyl alcohol and then add acetone again.

Concentrate the combined extracts to a syrup (volume about 150 c.c.) in a vacuum, add 100 c.c. of methyl alcohol and then 500 c.c. of acetone. Decant the liquid from the precipitate produced and extract the latter with a small amount of methyl alcohol. Add acetone to the extract, decant and combine the two solutions. Add to the methyl alcohol-acetone solution thus obtained an equal volume of freshly distilled, dry ether and pour off from the syrup which is precipitated (solution I). Now extract the residual syrup again with methyl alcohol and treat with acetone and ether as described above so that eventually solution II is obtained. Combine I and II, concentrate to a syrup in a vacuum, transfer to a large flat-bottomed crystallising basin, and leave until the next day in a desiccator containing sulphuric acid and granulated sodium hydroxide. As drying proceeds large well-formed crystals of ascorbic acid separate. Free them from mother liquor by washing with a little methyl alcohol, collect them by filtration with suction, wash them with acetone and dry in a vacuum. Up to this point the whole operation occupies, on the average, 3 days.

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[1] Szent-Györgyi and Svirbely, *Biochem. J.*, 1933, 27, 279.

By adding to the mother-liquors an excess of acetone and ether and treating as described above a second crop of crystals can be obtained.

The product consists of beautiful, pale yellow crystals having m.p. 187°–189°. From 10 litres of the juice, 6·5 g. of material are obtained, 5–6 g. in the first crop of crystals. In 65-day tests 0·5 mg. of the product protects guinea-pigs from scurvy.

Further Purification.—Dissolve the crystals with gentle warming in a mixture of three parts of methyl alcohol and two parts of diethylene oxide (dioxan) using 5 c.c. of liquid per gramme of ascorbic acid. Concentrate the liquid to small volume in a vacuum and leave overnight in the refrigerator. Wash the crystals with acetone and dry over sulphuric acid.

The product is now in the form of beautiful, white crystals which melt sharply at 192° ; $[a]_D^{20°} = +24°$.

l-Ascorbic acid was first obtained synthetically in good yield by Reichstein and Grüssner.[1]

Whilst ascorbic acid is to be regarded as a physiological *transformation product* of the carbohydrates, other substances having great physiological activity which have proved to be complex *compounds* containing carbohydrate residues, have recently been investigated. A large measure of success has been attained in elucidating their structures. Such compounds are, for example, the fermentation co-enzyme, cozymase (see p. 241) adenylic acid and adenyl pyrophosphate which are related to the nucleic acids (*q.v.*) and play an important part in the metabolism of muscle.

Recent investigations have shown that most proteins also contain certain carbohydrates as characteristic constituents of their molecules.[2]

Vitamin-B$_2$ (*lactoflavin* or 6 : 7-dimethyl-9-*d*-ribityl-*iso*-alloxazine) which has recently been synthesised by Karrer[1] and Kuhn[2] likewise contains a carbohydrate component which has a decisive effect on the activity. Karrer and Kuhn also elucidated the structure of lactoflavin.

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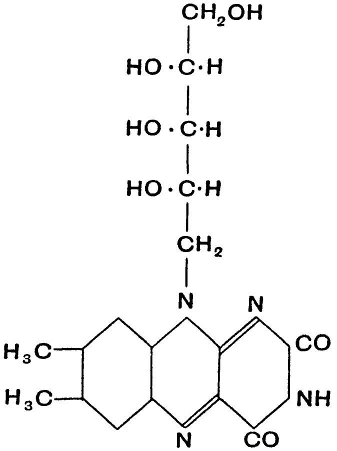
[1] *Helv. Chim. Acta.*, 1934, **17**, 311.

[2] Sørensen and Haugaard, *Biochem. Z.*, 1933, **260**, 247 ; Sørensen, *Biochem. Z.*, 1934, **269**, 271 ; Grassmann and Schleich, *Biochem. Z.*, 1935, **277**, 320.



Lactoflavin

It is also very probable that a phosphoric ester of lactoflavin combined with a specific protein component, which acts as the colloidal carrier of the vitamin, constitutes Warburg's yellow respiratory enzyme[3] (see p. 227).

ISOLATION OF LACTOFLAVIN FROM WHEY[4]

Although it is rather difficult to isolate lactoflavin with the means available in an instructional laboratory the procedure is nevertheless described here in order that the student may learn how this substance, which occurs in such minute amounts, is obtained.

Add to 300 litres of whey, obtained from fresh cow's-milk by treatment with rennet, 24·6 litres of concentrated hydrochloric acid and stir for 90 minutes with 2·4 kg. of fuller's earth. Allow to settle, draw off the liquid and wash the deposited material with distilled water until a test with silver nitrate shows the absence of chlorine-ions. Now stir for 90 minutes with a mixture of 2·7 litres of pyridine, 2·7 litres of methanol and 10·8 litres of distilled water. Remove the adsorbent with a centrifuge, concentrate the liquid to small volume in a vacuum and precipitate fuller's earth in colloidal solution by addition of two volumes of methanol. Again remove the precipitate in the centrifuge, remove the methanol by distillation in a vacuum and shake repeatedly with ether. Now add ten volumes of acetone and leave overnight. Next day, filter; concentrate in a vacuum to 350 c.c. and again adsorb the pigment by shaking for 45 minutes with 42 g. of the adsorbent "franconite KL". Wash repeatedly, and then elute the lactoflavin with a mixture of 75 c.c. of pyridine, 200 c.c. of methanol and 100 c.c. of water.

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[1] Von Euler, Karrer, Malmberg, Schöpp, Benz, Becker and Frei, *Helv. Chim. Acta*, 1935, **18**, 426, 522.

[2] Kuhn and co-workers, *Naturwiss.*, 1935, **23**, 260.

[3] Theorell, *Biochem. Z.*, 1935, **278**, 269. On the yellow respiratory enzyme see Warburg and Christian, *Naturwiss.*, 1932, **20**, 688, 980; *Biochem. Z.*, 1932, **254**, 438; 1932, **257**, 492.

[4] Kuhn, György and Wagner-Jauregg, *Ber.*, 1933, **66** [B], 1034.

At a pressure of 15 mm., concentrate the solution now to 75 c.c., add 2 c.c. of glacial acetic acid and 1 litre of acetone, remove with a centrifuge the precipitate which appears and again concentrate, this time to 8 c.c. As concentration proceeds, colourless precipitates appear repeatedly: remove them with the centrifuge. Add to the concentrated solution a saturated solution of picric acid which precipitates creatinine and other impurities leaving the lactoflavin in solution. Remove the excess of picric acid by extraction with ether in the dark. Concentrate the extracted solution in a vacuum. The pigment separates in crystalline form. Purify by recrystallising three times from boiling 2*N*-acetic acid. The yield is then 6 mg.

Lactoflavin crystallises in orange-brown needles which occasionally form sphaero-crystalline sheaves and between crossed Nicols exhibit almost complete extinction. When heated in a sealed tube in a copper block the crystals become increasingly darker from about 240° onwards and sinter and decompose at 267° (271° corrected).

Lactoflavin can be *synthesised* by the method of Karrer and his co-workers.[1]

Sodium hydrosulphite very readily reduces lactoflavin to the corresponding leuco-*compound* from which the yellow pigment with its green fluorescence is re-formed by the action of atmospheric oxygen.[2]

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[1] Von Euler, Karrer, Malmberg, Schöpp, Benz, Becker and Frei, *Helv. Chim. Acta*, 1935, 18, 522.

[2] Kuhn and Weygand, *Ber.*, 1934, 67 [B], 1409.

In view of the great importance of the carbohydrates and of the important part which they play as constituents or mother-substances of nucleic acids, proteins, vitamins, co-enzymes and so on, it is essential for the student who carries out any practical biochemical work to have accurate knowledge of the methods used for detecting, distinguishing and determining carbohydrates.

DETECTION AND DETERMINATION OF CARBOHYDRATES

A. Colour Reactions

1. **Reaction of Molisch.**—Add to 0·5 c.c. of the solution to be tested one to two drops of a 10 per cent alcoholic solution of α-naphthol and add concentrated sulphuric acid so that it forms a layer beneath the mixture. A violet-coloured ring is produced. This test is very delicate. It is positive with all carbohydrates except glucosamine. Glycuronic acid gives a green colour.

2. **The Naphthoresorcinol Test.**[1]—Warm the sugar for a few minutes with an equal amount of naphthoresorcinol in a mixture of equal parts of water and concentrated hydrochloric acid. A substance characteristically coloured according to the carbohydrate concerned is produced. (The coloured substance may have to be extracted with ether.)

3. **Selivanov's Resorcinol Reaction.**—Dissolve the sugar in 12 per cent hydrochloric acid and boil for 20 seconds (not longer !) with about half as much resorcinol. A red colour is produced in the case of ketoses (production of hydroxymethylfurfural). Weehuizen's modification : Evaporate the solution to a syrup, add ethyl alcohol, which has been saturated at 0° with hydrogen chloride, and shake at room temperature with resorcinol. Ketoses give a cherry-red colour after 3 minutes.

4. **Tollens' Orcinol Test.**—Procedure of Tillmanns and Philippi.[2] Heat for about 10 minutes on the water-bath a mixture of 15 c.c. of sulphuric acid (six volumes of concentrated acid to four volumes of water), 1 c.c. of the sugar solution and 0·5 c.c. of orcinol solution (2 g. of orcinol in 100 c.c. of 1 : 3 sulphuric acid). A yellow to orange-red colour is produced. With the help of a step photometer the depth of colour may be measured and the test used to distinguish

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[1] Tollens and Rorive, *Ber.*, 1908, 41, 1783.

[2] *Biochem. Z.*, 1929, 215, 40.

and determine various sugars belonging to different groups in the presence of each other. In this connexion consult the paper on the subject by Sørensen and Haugaard.[1] With amino-sugars the test is negative. They are detected by means of Ehrlich's reagent.

5. **Ehrlich's Reagent.**—*p*-Dimethylaminobenzaldehyde gives a characteristic colour with acetylglucosamine. In order that non-acetylated glucosamine may also be determined, a method of acetylating even minute amounts of the amino-sugar in the presence of other sugars has been developed.[2] Acetylglucosamine can then be determined colorimetrically in the following way:[3] Pour into a 10 c.c. measuring cylinder a solution of N-acetylglucosamine containing 0·1–1·0 mg. per c.c. of the sugar. If the volume of the solution is less than 1 c.c., dilute to 1 c.c. with water. Now add 0·1 c.c. of 0·5 per cent sodium carbonate solution, heat on a boiling water-bath for 5 minutes and cool. Prepare standard solutions in the same way. Then run in glacial acetic acid from a burette until the volume of liquid in the cylinder is about 8·0 c.c.; add 1 c.c. of Ehrlich's reagent (2 g. of dimethylaminobenzaldehyde in 100 c.c. of glacial acetic acid and 5 c.c. of concentrated hydrochloric acid); and make up to 10 c.c. with glacial acetic acid. The red colour is stable for about an hour.

B. REDUCTION REACTIONS

Carbohydrates containing free carbonyl groups give a series of reactions which depend on the capability for oxidation of this group.

The following tests are suitable for detecting reducing sugars:

1. **Trommer's Test.**—Treat the sugar solution with excess of alkali and cautiously add dilute copper sulphate solution as long as the precipitate first formed re-dissolves. Heat slowly to boiling. Yellow to red cuprous oxide separates. Do not add too much copper sulphate!

2. **Böttger's Test.**—Warm the sugar solution with Nylander and Almen's solution (2 g. of bismuth subnitrate and 4 g. of sodium potassium tartarate in 100 c.c. of 8 per cent sodium hydroxide solution). Black oxide of bismuth or bismuth metal is precipitated.

The following methods have proved suitable for determining reducing sugars:

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[1] *Biochem. Z.*, 1933, **260**, 247; 1934, **269**, 271.

[2] Zuckerkandl *et al.*, *Biochem. Z.*, 1931, **236**, 19.

[3] Morgan and Elson, *Biochem. J.*, 1934, **28**, 988.

3. **Titration with Fehling's Solution.**—The method depends on the principle of the Trommer test but is so carried out that, by addition of sodium potassium tartarate, the copper hydroxide is held in solution as a complex compound. For this reason also Fehling's solution is better for use in determining reducing sugars. Two solutions are used. They can be kept; but only when stored in separate vessels.

Solution I. Dissolve 34·639 g. of pure crystalline copper sulphate in 500 c.c. of water.

Solution II. Dissolve 173 g. of purest sodium potassium tartarate in a little water, add 100 c.c. of a solution containing 50 g. of sodium hydroxide and make up to 500 c.c.

For use mix equal parts of I and II. 20 c.c. of this solution correspond to 0·1 g. of glucose.

Procedure.—Pour exactly 20 c.c. of Fehling's solution into a basin and dilute with 80 c.c. of water. Then heat the solution to boiling and from a burette run in part of the solution of which the sugar content is to be determined. Boil for a few seconds and, if the solution remains blue, add a further quantity of the sugar solution. Repeat this procedure until the supernatant liquid has become colourless but not yet yellow. Red Cu_2O is deposited. The amount of sugar solution required to reach this state contains 0·1 g. of glucose. The method is rapid; but not very accurate, however.

4. **Titration by Bertrand's Method.**—This delicate method which is frequently employed in biochemical laboratories is likewise based on the reduction of the copper sulphate of Fehling's solution. In this case, however, the cuprous oxide produced serves further to reduce ferric sulphate and the amount of ferrous salt thus produced, determined by titration with permanganate, serves as a measure of the quantity of sugar which reacts. The reduction of the copper sulphate by the sugar is not strictly quantitative and the ratio—moles of copper sulphate reduced : moles of reducing sugar—depends on the nature of the sugar and the experimental conditions. Accordingly, in so far as duration of boiling and so on is concerned, it is necessary to adhere strictly to the instructions given. Tables have been constructed empirically for each sugar: in them the amount of sugar corresponding to each amount of Cu can be sought.[1]

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[1] Such tables are given, for example, in *Handbook of Chemistry and Physics*, 21st Ed., pp. 970-974 (Cleveland, Ohio, 1936).

The following table is intended for use in the calculation of glucose content from the amount of Cu: corresponding amounts are given in the adjacent columns.

Glucose in mg	Cu in mg	Glucose in mg	Cu in mg	Glucose in mg	Cu in mg.	Glucose in mg	Cu in mg.
10	20·4	33	64·6	56	105·8	79	144·5
11	22·4	34	66·5	57	107·6	80	146·1
12	24·3	35	68·3	58	109·3	81	147·7
13	26·3	36	70·1	59	111·1	82	149·3
14	28·3	37	72·0	60	112·8	83	150·9
15	30·2	38	73·8	61	114·5	84	152·5
16	32·2	39	75·7	62	116·2	85	154·0
17	34·2	40	77·5	63	117·9	86	155·6
18	36·2	41	79·3	64	119·6	87	157·2
19	38·1	42	81·1	65	121·3	88	158·8
20	40·1	43	82·9	66	123·0	89	160·4
21	42·0	44	84·7	67	124·7	90	162·0
22	43·9	45	86·4	68	126·4	91	163·6
23	45·8	46	88·2	69	128·1	92	165·2
24	47·7	47	90·0	70	129·8	93	166·7
25	49·6	48	91·8	71	131·4	94	168·3
26	51·5	49	93·6	72	133·1	95	169·8
27	53·4	50	95·4	73	134·7	96	171·4
28	55·3	51	97·1	74	136·3	97	173·1
29	57·2	52	98·9	75	137·9	98	174·6
30	59·1	53	100·6	76	139·6	99	176·2
31	60·9	54	102·3	77	141·2	100	177·8
32	62·8	55	104·1	78	142·8		

Solutions required:

I. Dissolve 40 g. of pure crystalline copper sulphate in 1 litre of water.

II. Dissolve 200 g. of sodium potassium tartarate and 150 g. of sodium hydroxide in water and dilute to 1 litre.

III. Dissolve 50 g. of ferric sulphate (free from $Fe^{\cdot\cdot}$!) in water, add 200 c.c. of concentrated sulphuric acid and dilute to 1 litre.

IV. Dissolve 5 g. of potassium permanganate in water and dilute to 1 litre. Standardise the solution with ammonium oxalate. 1 c.c. of 0·1 N-$KMnO_4$ corresponds to 6·36 mg. of Cu.

Procedure.—Add to the sugar solution (containing about 10–90 mg. of sugar) in a conical flask 20 c.c. each of solutions I and II, heat to boiling and continue boiling for 3 minutes. Then allow the Cu_2O produced to settle completely. (The supernatant liquid must still be blue.) Filter through a Jena glass filter, wash the precipitate with warm water until the wash-liquor is no longer alkaline (keep the precipitate always covered with water!) and by adding about 20 c.c. of solution III re-dissolve the precipitate on the filter collecting the solution in the filter flask. Wash out the filter carefully adding some more ferric sulphate solution if dissolution of the cuprous oxide is incomplete and titrate in the filter flask with solution IV until the green colour has given place to a pink.

5. **Titration by the Method of Willstatter and Schudel.**[1]—This method serves for the determination of free aldoses which are oxidised by hypoiodite to aldonic acids according to the equation :

$$CH_2OH\cdot(CHOH)_4\cdot CHO + I_2 + 3NaOH$$
$$= CH_2OH\cdot(CHOH)_4\cdot COONa + 2NaI + 2H_2O.$$

The special advantage of this reaction, which proceeds stoichiometrically, is that, under the experimental conditions, ketoses remain unattacked. Consequently, it is possible to determine aldoses in the presence of ketoses.

Treat the aldose with from 1·5 to 4 times the requisite amount of 0·1 *N*-I and add drop by drop with vigorous shaking at room temperature 1·5 times as much 0·1 *N*-NaOH as is equivalent to the iodine. Then leave to stand for 10–15 minutes (20 minutes when the amount of sugar is very small). Make slightly acid with dilute sulphuric acid and titrate with thiosulphate using starch as indicator.

6. **Determination by the Method of Hagedorn and Jensen.**[2]—This is, in the first place, a method for the determination of sugar in blood. The sugar reduces an alkaline ferricyanide solution to ferrocyanide which is precipitated as zinc salt. The excess of ferricyanide is iodometrically determined :

$$2K_4[Fe(CN)_6] + 3ZnSO_4 \longrightarrow K_2Zn_3[Fe(CN)_6]_2 + 3K_2SO_4$$
$$2H_3[Fe(CN)_6] + 2HI \longrightarrow 2H_4Fe(CN)_6 + I_2.$$

The following solutions are required :

I. Aqueous solution of 1·65 g. of potassium ferricyanide (A.R.) and 10·6 g. of Na_2CO_3 diluted to 1000 c.c. (Keep in the dark.)

II. Potassium iodide-zinc sulphate-sodium chloride solution. 5 g. of potassium iodide, 10 g. of purest zinc sulphate free from iron and 50 g. of sodium chloride dissolved to make 200 c.c. of solution. Keep on hand a large amount of the solution without the potassium iodide and add small amounts of potassium iodide as required immediately before use.

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[1] *Ber.*, 1918, 51, 780.

[2] *Biochem. Z.*, 1923, 135, 46 ; 1923, 137, 92.

III. Acetic acid. 3 c.c. of Kahlbaum's iron-free acetic acid diluted to 100 c.c. with water.

IV. Starch solution. 1 per cent in saturated potassium chloride solution.

V. Thiosulphate solution 0·005*N*. 0·7 g. of sodium thiosulphate in 500 c.c. of water. Standardise with potassium iodate. Make up a solution of 0·3566 g. of purest anhydrous potassium iodate to 2 litres.

VI. For deproteinisation of solutions containing protein (*e.g.* blood) (*a*) 0·1*N*-sodium hydroxide solution and (*b*) 0·45 per cent zinc sulphate solution. (*a*) and (*b*) must be freshly prepared every 8 days from 2*N*-NaOH and 45 per cent zinc sulphate solution.

AMOUNTS OF GLUCOSE CORRESPONDING TO TITRATION VALUES WHEN 0·1 C.C. OF BLOOD IS USED[1]

MILLIGRAMMES OF GLUCOSE IN 0·1 C.C. OF BLOOD

c c of 0 005*N*-$Na_2S_2O_3$	Hundredths of one c.c. of 0 005*N*-Sodium Thiosulphate $Na_2S_2O_3$									
	0	1	2	3	4	5	6	7	8	9
0·0	0·385	0·382	0·379	0·376	0·373	0·370	0·367	0·364	0·361	0·358
0·1	0·355	0·352	0·350	0·348	0·345	0·343	0·341	0·338	0·336	0·333
0·2	0·331	0·329	0·327	0·325	0·323	0·321	0·318	0·316	0·314	0·312
0·3	0·310	0·308	0·306	0·304	0·302	0·300	0·298	0·296	0·294	0·292
0·4	0·290	0·288	0 286	0·284	0·282	0·280	0·278	0·276	0·274	0·272
0·5	0·270	0·268	0·266	0·264	0·262	0·260	0·259	0·257	0·255	0·253
0·6	0·251	0·249	0·247	0·245	0·243	0·241	0·240	0·238	0·236	0·234
0·7	0·232	0·230	0·228	0·226	0·224	0·222	0·221	0·219	0·217	0·215
0·8	0·213	0·211	0·209	0·208	0·206	0·204	0·202	0·200	0·199	0·197
0·9	0·195	0·193	0·191	0·190	0·188	0·186	0·184	0·182	0·181	0·179
1·0	0·177	0·175	0·173	0·172	0·170	0·168	0·166	0·164	0·163	0·161
1·1	0·159	0·157	0·155	0·154	0·152	0·150	0·148	0·146	0·145	0·143
1·2	0·141	0·139	0·138	0·136	0·134	0·132	0·131	0·129	0·127	0·125
1·3	0·124	0·122	0·120	0·119	0·117	0·115	0·113	0·111	0·110	0·108
1·4	0·106	0·104	0·102	0·101	0·099	0·097	0·095	0·093	0·092	0·090
1·5	0·088	0·086	0·084	0·083	0·081	0·079	0·077	0·075	0·074	0·072
1·6	0·070	0·068	0·066	0·065	0·063	0·061	0·059	0·057	0·056	0·054
1·7	0·052	0·050	0·048	0·047	0·045	0·043	0·041	0·039	0·038	0·036
1·8	0·034	0·032	0·031	0·029	0·027	0·025	0·024	0·022	0·020	0·019
1·9	0·017	0·015	0·014	0·012	0·010	0·008	0·007	0·005	0·003	0·002

Procedure.—Pour into a boiling tube (1·5 × 15 cm.) 1 c.c. of 0·1*N*-NaOH and 5 c.c. of the zinc solution VI (*b*). A gelatinous precipitate of zinc hydroxide is produced. From a micro-pipette blow into the suspension 0·1 c.c. of blood, wash out the pipette twice with the mixture and heat the tube for 3 minutes in the boiling water-bath. Protein is thus precipitated and floats as a grey coagulum in the clear liquid. Filter through washed moist cotton wool in a filter funnel having a diameter of 3–4 cm., collecting the filtrate in a boiling tube (3 × 9 cm.) and wash with two 3 c.c. portions of warm water. Now add 2 c.c. of solution I and heat for 15 minutes in the boiling water-bath. Then cool, add 3 c.c. of solution II, mix, acidify with 2 c.c. of solution III and titrate the liberated iodine with the thiosulphate solution (V) after addition of two drops of solution IV. A table which shows the amount of glucose corresponding to the number of cubic centimetres of thiosulphate solution used will be found above. Blank determinations must be carried out in parallel and account taken of the quantities of glucose thus determined.

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[1] Hagedorn and Jensen, *Biochem. Z.*, 1923, 135, 46; 1923, 137, 92.

INSULIN

Insulin, the hormone of the pancreas, has a decisive effect on carbohydrate metabolism. The sources of the hormone are the so-called islets of Langerhans in the pancreas. If the pancreas is absent or if the function of the islets is damaged owing to pathological causes, the normal transformation of sugar does not occur and sugar accumulates in the blood (hyperglycaemia ; diabetes mellitus or " sugar disease "). The sugar content of the blood is rapidly decreased by parenteral administration of insulin. The sugar which disappears is partly converted into glycogen in the liver [1] and partly oxidised to CO_2 in the muscles,[2,3] as is indicated by the increase in the respiratory quotients.

Crystalline insulin, which was first prepared by Abel [4] and then, more simply, by Harington,[5] exhibits pronounced protein character. When it is hydrolysed known amino-acids are produced. It is believed that the peculiar properties of insulin are to be traced to an *active* group which is united to the protein. Recent investigations seem to show that this group is the disulphide group –S–S–, one S-atom being united to a protein-like carrier and the other to a smaller residue.[6]

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[1] Cori and Cori, *J. Biol. Chem.*, 1926, **70**, 557.

[2] Lesser and Bissinger, *Biochem. Z.*, 1926, **168**, 398.

[3] Best, Hoet and Marks, *Proc. Roy. Soc.*, 1926, **100 B**, 32, 72.

[4] *J. Pharm. Exp. Ther.*, 1927, **31**, 65.

[5] *Biochem. J.*, 1929, **23**, 384.

[6] Freudenberg and Wegmann, *Z. physiol. Chem.*, 1935, **233**, 159.

A very active sample of insulin can be obtained by the following procedure which is described as an example of such methods.[1]

Remove the fat from 3 kg. of fresh pancreas (pig or ox) and mince. Add to the minced material 1500 c.c. of distilled water containing 35 c.c. of concentrated sulphuric acid. Stir well for 15 minutes, add 7·5 litres of alcohol, and stir well again for 1 hour with a mechanical stirrer. Now transfer to a centrifugal separator, spin, reject the residue and measure the p_H of the solution. The value should be 2·5–3·0. Pour through a large folded filter and for each litre of filtrate add 1·1 c.c. of sodium hydroxide solution (*d* 1·5). Concentrate the neutralised solution to one-tenth of its original volume by distillation under reduced pressure taking care that the temperature during distillation does not exceed 40°. The concentrated solution contains much fat. Remove this by extracting thrice with ether.

To the defatted solution add 22 per cent sodium chloride solution. A whitish precipitate which rises to the surface is produced. Siphon off the liquid and reject it, dissolve the precipitate in the original volume of water and precipitate again with 22 per cent sodium chloride solution. Now remove the liquid as completely as possible with a siphon or a centrifuge and dissolve the precipitate in 95 per cent alcohol using 5·6 c.c. for each litre of the original alcoholic extract. Filter or, better, separate in a centrifuge and precipitate the insulin by addition of two volumes of ether. Then allow the precipitate to settle well and separate the supernatant alcohol-ether solution in a centrifuge. Dry the precipitate well in a desiccator over sulphuric acid, grind to a fine powder in an agate mortar and weigh. The material thus obtained contains many impurities, chiefly proteins which are removed by precipitation of the insulin at the iso-electric point. The iso-electric point of insulin is at p_H 5·5–5·6. Dissolve the powder in water using 22 c.c. for each litre of the original alcoholic extract. Now add, drop by drop, *N*-sodium hydroxide solution, keeping a check on the p_H and taking care that it does not pass 5·5. At this p_H a whitish precipitate separates. Allow it to settle, separate it from the liquid in a centrifuge and dry it with absolute alcohol and ether. The material thus produced is relatively very pure insulin. It contains about 12–15 insulin units per mg. One of the authors

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[1] Blatherwick, Bischoff, Maxwell, Berger and Sahyun, *J. Biol. Chem.*, 1927, 72, 57.

(Grassmann) has frequently succeeded in obtaining crystalline insulin by Abel's method[1] from material prepared in this way.

By the method described below determine the insulin content of the material obtained.

DETERMINATION OF INSULIN CONTENT

In order to measure the activity of the material it is injected into rabbits and the consequent reduction in the sugar content of the blood is observed during a determined period (determination of sugar by the method of Hagedorn and Jensen, see p. 76). The League of Nations or international unit[2] is employed in measuring the activity of insulin preparations. An international unit is the amount which reduces the sugar content of the blood of a normal rabbit weighing about 2 kg. to the threshold of the onset of convulsions, namely 0·045 per cent, within 3 hours, the rabbit having fasted for 24 hours before the experiment. Many investigators, however, employ their own units which are then expressed in terms of the international unit.

To test the material prepared, inject subcutaneously into a fasting rabbit about 0·1 mg. of insulin dissolved in 1 c.c. of 0·01 N-hydrochloric acid and measure the reduction in the sugar content of the blood drawn from the vein in the ear. Determine the sugar by the method of Hagedorn and Jensen (see p. 76). Make the first determination shortly before the injection and the others 1, 2 and 4 hours after it. If convulsions should set in at once inject subcutaneously 20 c.c. of 20 per cent glucose solution so that the rabbit becomes normal again as rapidly as possible.

For an account of the exact procedure in the biological determination of insulin see the paper by Freudenberg and Dirscherl.[3]

3. NUCLEINS

NUCLEOPROTEINS

Nucleoproteins are compounds of proteins with nucleic acids, *i.e.* compounds which possess the essential properties of nucleic acids in addition to those of proteins. Thus they contain purines or pyrimidines, carbohydrates and organically bound phosphoric acid.

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[1] Abel, *loc. cit.*

[2] *The Biological Standardisation of Insulin* (Publications of the League of Nations, III. *Health*, 1926, 3, 7); E. Knaffl-Lenz, *Arch. exp. Path. Pharm.*, 1928, 135, 258.

[3] *Z. physiol. Chem.*, 1929, 180, 212.

They are found widely distributed in the animal and vegetable kingdoms particularly in the nuclei of cells. They have been isolated from the spermatozoa, thymus, pancreas, brain, adrenals, thyroid gland, lymph glands, bone marrow, leucocytes, placenta, liver, mammary glands and also from the muscle and blood of animals. In the vegetable kingdom the best starting material for their preparation is yeast.

They are usually isolated by extracting with dilute alkalis, water and sodium chloride solution, and precipitating with alcohol or occasionally by cautious addition of dilute acid. They are white, amorphous powders having an acid reaction and are easily denatured and coagulated by heat.

POLYNUCLEOTIDES

The protein components of the polynucleotides are usually eliminated by mild alkaline treatment leaving the alkali salts of the nucleic acids. The free nucleic acids can then be obtained from their salts by precipitation with acetic acid-alcohol mixture.[1]

" Nucleic acids " possess the structural peculiarities of the polynucleotides : they consist of a limited number of mononucleotides united in a manner so far not known in detail. These mononucleotides are composed of 1 mole of purine or pyrimidine, 1 mole of pentose and 1 mole of phosphoric acid. The tetranucleotides seem to play a predominating rôle in nature. Since the nucleic acids contain sugar they give a positive pentose reaction when boiled with orcinol and hydrochloric acid or with phloroglucinol and hydrochloric acid.

Preparations from yeast differ considerably both in chemical behaviour and analytical composition from the nucleic acids obtained from the glands of animals. Even at room temperature *yeast nucleic acid* is decomposed by dilute alkali into its individual nucleotides : adenylic acid, guanylic acid, cytidylic acid and uridylic acid whilst *thymonucleic acid* resists such treatment. Another fundamental difference concerns the structure of the carbohydrate component. The carbohydrate of yeast nucleic acid is *d*-ribose, that of thymonucleic acid is (chiefly) 2-deoxy-*d*-ribose. Accordingly, polynucleotides of the yeast nucleic acid type are termed ribopolynucleotides in contrast to the deoxyribopolynucleotides of thymonucleic acid. The types are distinguished by eliminating the phosphoric acid from the mononucleotides leaving purine-pentose or pyrimidine-pentose compounds (nucleosides) of various types, namely, ribosides and de-oxyribosides.

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[1] Jones, *Nucleic Acids* (London, 1920).

Preparation of Yeast Nucleic Acid.[1]—The preparation of pure yeast nucleic acid, especially in the laboratory, is rather difficult. The chemical firm, C. F. Böhringer und Söhne, in Mannheim, supplies good quality material. This is a white powder, difficultly soluble in water, dilute acids and ether but easily soluble in alkalis. *Tritico-nucleic acid* obtained from wheat germ is practically identical with yeast nucleic acid.

Preparation of Thymonucleic Acid.[2]—Clean 1 kg. of fresh thymus and boil with water containing a little acetic acid until the solid becomes quite hard. Chop finely and stir thoroughly with 2 litres of boiling water containing 200 g. of sodium acetate. Now add 100 c.c. of 33 per cent sodium hydroxide solution and heat under reflux on the boiling water-bath for from 30 minutes to 2 hours. Neutralise with about 150 c.c. of 50 per cent acetic acid, filter, concentrate the filtrate to about 500–1000 c.c., cool to 40° and add an equal volume of alcohol. Dissolve the precipitated sodium salt in 500 c.c. of water, heat on the water-bath until the turbid liquid has deposited a precipitate and become clear again; filter and add alcohol again. Repeat the dissolution and precipitation until the alcohol produces a precipitate only after addition of a little sodium acetate. Treat the sodium salt of thymonucleic acid thus obtained with alcohol and dry in a desiccator. The product is a fine white powder which resembles yeast nucleic acid in regard to its solubility. To prepare the free acid (which is very easily decomposed, however), pour the solution of the sodium salt into three volumes of cold alcohol containing hydrochloric acid (2 c.c. of concentrated acid to 98 c.c. of alcohol). Collect the precipitate by filtration, wash with alcohol until the filtrate is free from Cl′ and finally wash with ether. Yield: 30–35 g.

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[1] Altmann, quoted by Steudel and Peiser, " Nucleinkörper " in Klein's *Handbuch der Pflanzenanalyse*, Bd. 4, III. Teil, p. 432 (Vienna, 1933); Osborne and Harris, *Z. physiol. Chem.*, 1902, **36**, 85; Slate, *Amer. J. Physiol.*, 1905, **13**, 464; Levene, *Biochem. Z.*, 1909, **17**, 120; Clarke and Schryver, *Biochem. J.*, 1917, **11**, 319; Baumann, *J. Biol. Chem.*, 1918, **33**, 14.

[2] Neumann, *Arch. Anat. Physiol.*, 1898, *Physiol. Abteil.*, 374; Erganzungsband, 1899, 552; Feulgen, *Z. physiol. Chem.*, 1914, **90**, 261; 1914, **91**, 165; Levene, *Z. physiol. Chem.*, 1905, **45**, 370; *J. Biol. Chem.*, 1922, **53**, 441.

MONONUCLEOTIDES

It was mentioned above that the mononucleotides are composed of purine or pyrimidine, pentose and phosphoric acid, 1 mole of each, united in the form of a chain. Since they exhibit reducing properties only after hydrolysis it is assumed that the aldehyde group of the sugar is blocked. The basic complex is united as in a glucoside; ribose has a *furanose* structure [1] but in all probability deoxyribose has a *pyranose* structure.

When the base is separated from the nucleotide molecule ribose phosphoric acid is obtained. In the elucidation of the constitution, special importance attaches to the isomerides which arise through variation in the position of the phosphoric acid residue in the *d*-ribose molecule. H_3PO_4 may react with hydroxyl at the third or the fifth carbon-atom. The reasons for the existence of two riboadenylic acids are given on page 85. Corresponding deoxyribophosphoric acids have not been isolated, so that up to the present, the point of attachment of the phosphoric acid in the thymonucleotides is unknown.

The **ribonucleotides** are obtained by hydrolysis of yeast nucleic acid with dilute alkali followed by fractional crystallisation of the brucine salts.[2] If the attempt to obtain purine nucleotides is not made the pyrimidine nucleotides which are much more resistant can also be prepared from yeast nucleic acid by hydrolysis with dilute acid.

Guanylic Acid: $C_{10}H_{14}O_8N_5P \cdot 2H_2O$.—This acid is relatively simply prepared as follows [3]: Dissolve 500 g. of the sodium salt of yeast nucleic acid in 2 litres of water, cool to 0° and at this temperature add 200 c.c. of 33 per cent sodium hydroxide solution, which has likewise been cooled to 0°. Leave to stand for about 10 minutes in the cold and then separate by filtration from precipitated calcium phosphate. Now add 200 g. of sodium acetate and 6 litres of alcohol which precipitates the crude product. Repeat this precipitation which serves for purification. Then dissolve the precipitate in 50 c.c. of ice-cold water, neutralise with glacial acetic acid and add 100 g. of sodium acetate. Not a trace of sodium guanylate is precipitated by this procedure. Make the solution slightly alkaline and leave at room temperature for 24 hours. The whole of the guanylic acid separates as secondary

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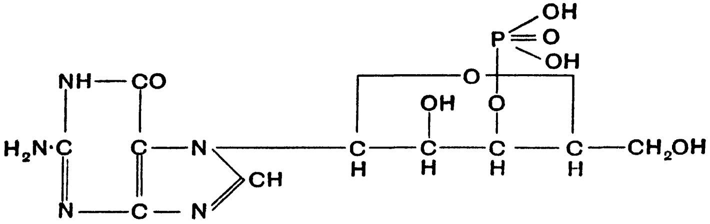
[1] Bredereck, *Ber.*, 1932, 65 [B], 1830.

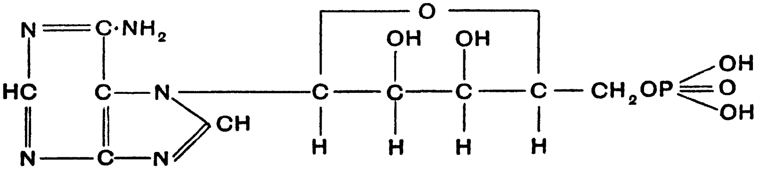
[2] Levene, *J. Biol. Chem.*, 1918, 33, 425; 1919, 40, 415.

[3] Steudel and Peiser, *Z. physiol. Chem.*, 1922, 120, 292.

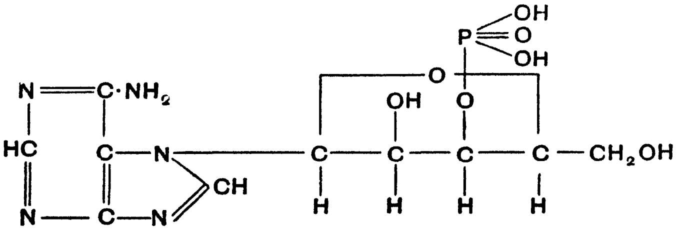


sodium salt. Reprecipitate this twice from hot 33 per cent sodium acetate solution (1 litre each time), dissolve in 500 c.c. of 2*N*-sodium hydroxide solution and add alcohol. Rub with a glass rod. The crystalline tertiary sodium salt of guanylic acid separates. Yield : 87 g.

Adenylic Acid: $C_{10}H_{14}O_7N_5P$.—There are two riboadenylic acids, namely *yeast* or h-*adenylic acid* and *muscle* or t-*adenylic acid.*



Muscle Adenylic Acid



Yeast Adenylic Acid

These differ considerably in their physical and chemical properties (solubility, optical rotation, hydrolysis), especially, however, in their behaviour towards certain enzymes. Muscle adenylic acid is deaminated by an enzyme of muscle to *t*-inosic acid whilst *h*-adenylic acid remains completely unchanged by this enzyme. These differences are the result of the different positions of the phosphoric acid residue in the sugar molecule. When this residue is removed by dephosphorylation, subsequent deamination of the *h*-adenosine which remains gives hypoxanthosine. Hypoxanthosine is identical with the inosine of muscle. Closer study of the structures shows that in muscle adenylic acid the phosphoric acid residue is attached to the end C-atom of the pentose, whilst in yeast adenylic acid it is attached to the third C-atom.

Since the hydrolysis of the glucoside links of *h*-adenylic acid and guanylic acid (as well as of its deamination product xanthylic acid) leads to the same 3-ribosephosphoric acid, it follows that the phosphoric acid residue of guanylic acid is also in the 3-position.

Preparation of Yeast Adenylic Acid.[1]—Add to the combined filtrate and wash liquor left after separation of the guanylic acid (see above) neutral lead acetate until separation of a precipitate ceases. Wash the precipitate well with water and free it from lead by treatment with hydrogen sulphide. Neutralise with ammonia, concentrate by distillation under reduced pressure and treat the residue with an equal volume of alcohol which produces an oily precipitate. Repeat this precipitation twice. Most of the oil redissolves. A small flocculent precipitate of the ammonium salt of guanylic acid remains undissolved. Reconvert the material in the various filtrates into lead salt, remove the lead with hydrogen sulphide and concentrate the solution of the free acid by distillation under reduced pressure at about 40°. A copious separation of crystals (needles) sets in. When dried they weigh 57·5 g. Melting point 195°.

Adenylpyrophosphoric Acid (adenosine triphosphate): $C_{10}H_{14}O_7N_5P \cdot H_4P_2O_7 \cdot H_2O$.—Another nucleotide, namely, adenylpyrophosphoric acid, occurs in all animal cells and particularly in striped muscle. It contains adenylic acid and pyrophosphoric acid in loose combination. Frequently these two acids separate spontaneously and they are separated particularly readily by enzymes. According to Lohmann[2] there are in adenylpyrophosphoric acid three molecules of phosphoric acid united with each other in ester-like combination and attached to the fifth C-atom of ribose. The acid is prepared from the muscle of the frog or rabbit by Lohmann's method.[1]

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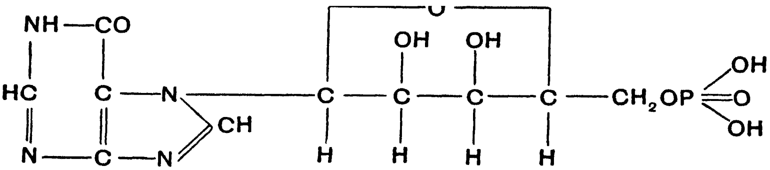
[1] Steudel and Peiser, *Z. physiol. Chem.*, 1923, 127, 262.

[2] *Biochem. Z.*, 1935, 282, 109, 120.

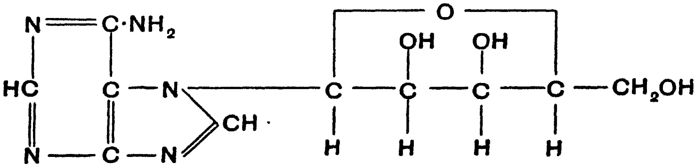
Adenylpyrophosphoric acid acts as co-enzyme in glycolysis. It is not identical with cozymase, the co-enzyme of alcoholic fermentation, although this co-enzyme is likewise an adenine nucleotide. In this connexion see p. 242.

l-**Inosic acid,** $C_{10}H_{13}O_8N_4P$, is produced from *l*-adenylic acid by enzymic deamination or by treatment with nitrite and glacial acetic acid. The best preparative method of obtaining inosic acid is from meat extract [2] in which it was first discovered long ago by Liebig.[3]



Nucleosides

The compounds which are obtained from the nucleotides by elimination of phosphoric acid are termed nucleosides. They are glucoside-like compounds containing a sugar united to a purine or pyrimidine base. The phosphoric acid can be eliminated by alkaline, neutral or acid hydrolysis. Enzymes from the intestinal mucous membrane and from the liver bring about enzymic hydrolysis and can be used in the preparation of nucleosides.[4]



Adenosine, $C_{10}H_{13}O_4N_5$.—Melting point 229°. This occurs in cardiac and skeletal muscle, in milk, blood and urine. It is a compound of adenine with *d*-ribose.

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[1] *Biochem. Z.*, 1931, **233**, 460.

[2] Haiser, *Monatsh.*, 1895, **16**, 190; 1908, **29**, 157.

[3] *Annalen*, 1847, **62**, 317.

[4] Bielschowsky and Klein, *Z. physiol. Chem.*, 1932, **207**, 202.

4. LIPINS

An exact definition of the term " lipins " cannot yet be given. In general they include, in addition to the fats, fat-like substances which can be extracted with organic solvents and play an important rôle in the animal and vegetable organism.

FATS

In general, fats are the glyceryl esters of higher fatty acids, in particular of palmitic acid $C_{16}H_{32}O_2$ and of stearic acid $C_{18}H_{36}O_2$, and also of arachidic $C_{20}H_{20}O_2$, behenic $C_{22}H_{44}O_2$ and lignoceric acid $C_{24}H_{48}O_2$. The chief unsaturated acid concerned in the production of fats is oleic acid (Δ^9-stearic acid[1]). Erucic acid (Δ^{13}-behenic acid), nervonic acid (Δ^{15}-lignoceric acid), linoleic acid (Δ^{12}-oleic acid), linolenic acid (Δ^{15}-linoleic acid,) ricinoleic acid (12-hydroxyoleic acid) and other higher unsaturated fatty acids also occur in the fats.

These acids may be combined with glycerol in various ways. Thus mono-, di- and tri- glycerides are produced when one, two or three of the hydroxyl groups of glycerol respectively are esterified. Simple glycerides are those containing only one acid whilst mixed glycerides contain more than one. Position isomerism can occur according to whether the substitution occurs in the α-, α' or β-position so that further possibility of variation arises.

The natural fats are for the most part mixed triglycerides. Their consistency at room temperature gives a rough idea of their chemical composition. Liquid or semi-liquid fats frequently contain a high proportion of unsaturated acids. Other data, such as those now enumerated, permit more accurate characterisation:

The **acid or neutralisation value** which is the number of milligrammes of KOH required to neutralise the *free* fatty acids in one gramme of fat.

The **saponification value** which is the number of milligrammes of KOH required to neutralise the free organic acids *and* to hydrolyse the esters and lactones in one gramme of fat.

The **ester value** is the number of milligrammes of KOH required to hydrolyse the esters in one gramme of fat. (The ester value is the difference between the saponification and acid values.)

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[1] Δ^9 signifies a double bond between C_9 and C_{10}, the COOH-group being included.

The **iodine value** is the percentage of halogen, calculated as iodine, which can be added to a substance containing double bonds under certain conditions.

The **hydrogenation value** is the number of grammes of hydrogen required for the quantitative hydrogenation of 10,000 grammes of fat.

Methods of determining these values can be found in the special literature on the subject.[1]

" Oils " and semi-solid fats can be catalytically hydrogenated (technical process for hardening fats) using nickel as catalyst. The double bonds of the unsaturated acids become saturated as a result. The drying of oils through the production of oxides and peroxides or through polymerisation also depends on the reactivity of double bonds.

Fat Metabolism.—Fats undergo biological degradation as a result of the action of lipase (see p. 136), the first products being glycerol and fatty acids. The subsequent fate of these two components is obscure. In the animal organism, partial re-synthesis of the original fat occurs after absorption in the wall of the intestine. Another part of the products of degradation seems to be used in the synthesis of phosphorus-containing complexes (see pp. 89, 90) and of cholesteryl esters.

The actual shortening of the long carbon chains of the molecules of the fatty acids may occur in two ways : by *rupture* of *double bonds* produced where necessary by dehydrogenation [2] or by β-oxidation [3] which proceeds somewhat as follows :

$$R{\cdot}CH_2{\cdot}CH_2{\cdot}COOH \xrightarrow{-H_2} R{\cdot}CH{:}CH{\cdot}COOH \xrightarrow[-H_2]{+H_2O} R{\cdot}CO{\cdot}CH_2{\cdot}COOH$$

$$\xrightarrow{+H_2O} R{\cdot}COOH + CH_3{\cdot}COOH$$

(The probable correctness of the second hypothesis, namely, that two carbon-atoms at a time are removed from carbon chains is supported by the results of model experiments and by the fact that the molecules of most natural fatty acids contain an even number of carbon-atoms. β-Oxidation is occasionally preceded by ω-*oxidation* in which a methyl group at the end of a chain is attacked and a dicarboxylic acid is produced as an intermediate product.[1])

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[1] Lewkowitsch, *Chemical Technology and Analysis of Oils, Fats and Waxes,* 3 vols., 6th Ed. (London, 1921–1923); Leathes and Raper, *The Fats* (London, 1925); Leathes, *Lancet,* 1909, i. 593.

[2] Leathes, *Lancet,* 1909, i., 593.

[3] Knoop, *Beitr. chem. Physiol. Pathol.,* 1905, 6, 150 ; *Oxydationen im Tierkörper,* (Stuttgart, 1931).

Experimental evidence concerning the course of the biological synthesis of fatty acids has not yet been adduced. It is supposed, however, that aldol or acetoin condensations amongst aldehydes (acetaldehyde, trioses, hexoses) and subsequent dismutation of the condensation products to acid and alcohol are involved.

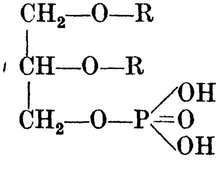
WAXES

The alcohols involved in the building up of waxes are higher members of the series in contrast to those which occur in most fats. Such higher alcohols are cetyl alcohol $C_{16}H_{33}OH$, lignoceryl alcohol $C_{24}H_{49}OH$, ceryl alcohol $C_{26}H_{53}OH$ and myricyl alcohol $C_{31}H_{63}OH$. The fatty acids of the waxes include, in addition to several well-known acids, cerotic acid $C_{26}H_{52}O_2$, melissic acid $C_{31}H_{62}O_2$ and lacceric acid $C_{32}H_{64}O_2$.

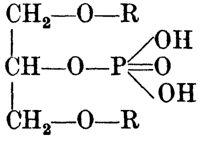
In the waxes of the coniferae there occur acids which possess both acidic and alcoholic properties and can unite with each other to form esters.

PHOSPHATIDE ACIDS

In the leaves of cabbage and spinach there occur glycerophosphoric acids having the following constitutions (R = a fatty acid residue):



α-Glycerophosphoric acid



β-Glycerophosphoric acid

These are intermediate products in the synthesis of the complex lipins known as phosphatides.

PHOSPHATIDES

The phosphatides form a group of compounds related to the fats and contain glycerol, fatty acid, phosphoric acid and a nitrogenous base.[1] Up to the present it has been possible with certainty to include the following groups of substances amongst the phosphatides : lecithins, kephalins, sphingomyelins.

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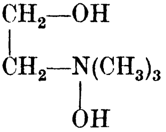
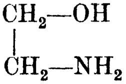
[1] Verkade *et al.*, *Z. physiol. Chem.*, 1933, 215, 225; *Biochem. J.*, 1934, 28, 31.



$$\begin{array}{l} CH_2\text{—}OH \\ | \\ CH_2\text{—}N(CH_3)_3 \\ \quad\quad\;\; | \\ \quad\quad\;\; OH \end{array}$$

Choline, the base in the case of the lecithins



$$\begin{array}{l} CH_2\text{—}OH \\ | \\ CH_2\text{—}NH_2 \end{array}$$

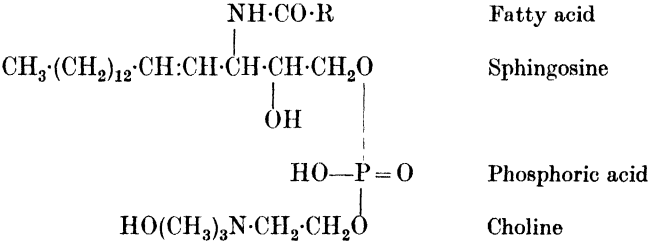
Colamine, the base in the case of the kephalins

The lecithins are the alcohol-soluble, the kephalins the alcohol-insoluble part of the phosphatide molecule.

The sphingomyelins, which derive their name from the dihydric, basic, unsaturated alcohol sphingosine, $C_{18}H_{37}O_2N$.

$$CH_3\cdot(CH_2)_{12}\cdot CH{:}CH\cdot CH(NH_2)\cdot CH(OH)\cdot CH_2OH$$

probably have the following constitution :



The following fatty acids have been encountered as parts of the phosphatide molecule : palmitic, stearic, nervonic, lignoceric.

Phosphatides occur in many animal and vegetable cells and tissues; in large amounts in nerves, in the adrenal glands, in cardiac muscle, in egg-yolk and in seeds.

CEREBROSIDES

Cerebrosides are substances built up from 1 molecule each of galactose, sphingosine and a higher fatty acid. They are isolated chiefly from brain and nerve tissue but also occur in other animal tissues and fluids. They have not yet been detected, with certainty, in plants.

On account of the great similarity which the various cerebrosides exhibit—only the fatty acid components differ—they can be separated from each other only with difficulty.

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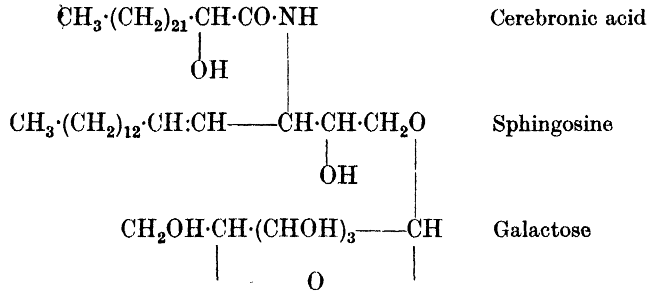
[1] Thierfelder and Klenk, *Die Chemie der Cerebroside und Phosphatide* (Berlin, 1930) ; H. and I. S. Maclean, *Lecithin and Allied Substances* ; *The Lipins*, 2nd Ed. (London, 1927).

Cerebrone or phrenosine $C_{48}H_{93}O_9N$.



Preparation of Cerebrone.[1]—Remove the blood and skin from brain (preferably human brain) and pass it not less than four times through the smallest size of perforated plate in a mincing machine. Place portions weighing 2·5 kg. each in large wide-necked flasks, cover each portion with about 3 litres of acetone and leave for 24 hours at room temperature, with frequent shaking. Filter with suction, using a large funnel, wash once with acetone and return the washed mass to the flasks. Repeat the treatment with acetone and filter as dry as possible with suction. Then pour over the material about 1500 c.c. of ether; leave for 24 hours with frequent shaking; filter and continue the extraction with ether until it ceases to acquire colour and leaves only a small residue on evaporation. (Four or five extractions are necessary.) Cool the ethereal extracts to 0° and remove by decantation and centrifugation the material which separates. Grind to a fine powder the material which has been exhaustively extracted with ether (this material weighs about 350 g. equivalent to 13–14 per cent of the fresh weight), pass the powder through a fine sieve, boil for 5 minutes with five volumes of 85 per cent alcohol and filter with suction while hot, using double filter paper on a large funnel. Cool the filtrate, filter off with suction the material which separates and use the filtrate for re-extraction. Repeat the extraction four times using the same amount of alcohol (usually four extractions suffice). Shake with ether the material which is deposited from the alcohol, separate again in a centrifuge and combine with the material separated already in the same way. Remove adherent ether by evaporation from the snow-white mass (the so-called protagon, weight

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[1] Kitagawa and Thierfelder, *Z. physiol. Chem.*, 1906, 49, 286; Loening and Thierfelder, *Z. physiol. Chem.*, 1910, 68, 464.

80–100 g. equivalent to 3–4 per cent of the fresh brain), and dissolve it in 75 per cent chloroform (100 g. of methyl alcohol added to about 500 c.c. of chloroform). Warm gently so as to bring about easy and complete dissolution. Remove paper fibres and other impurities by filtration and leave at room temperature in a closed flask. A thick, hard crust forms on the surface within 24 hours or less. Filter, cool the filtrate and, after filtration, recrystallise several times from 75 per cent chloroform-methyl alcohol the material which is deposited. Repeat this procedure until separation again occurs in the form of a hard, white crust on the surface of the clear liquid. Evaporate the mother liquors to dryness under reduced pressure and recrystallise the residue from the same solvent. The separation again occurs in the same characteristic way. Combine the various portions of the material, dissolve it in twenty-five to thirty parts of hot methyl alcohol containing 20 per cent of chloroform and cool. The precipitate which is produced still contains small amounts of phosphorus-containing material. Remove this material by dissolving the precipitate in hot methyl alcohol containing 10 per cent of chloroform and add hot solution of zinc reagent (obtained by suspending zinc hydroxide in methyl alcohol passing in ammonia and adding ammonium acetate) and boil until the turbidity first produced gives place to a flocculent mass. Now filter off this mass which is very rich in phosphorus. Cool the filtrate, filter off the precipitate which separates and re-dissolve it in methyl alcohol containing 10 per cent of chloroform. Reject the small amount of undissolved material which contains zinc and phosphorus. Cool the filtrate obtained on removing this material. Cerebrone separates usually in the amorphous state but occasionally chiefly in the form of shining crystalline plates. Microscopic examination shows that the crystals also contain amorphous impurities, however. Remove these by dissolving the material in hot methyl alcohol containing 20 per cent of chloroform and separating, by filtration through a hot water funnel, the fractions which are precipitated within certain limits of temperature, testing small samples of each fraction in order to discover the extent to which crystalline material can be obtained (completely, copiously, sparingly or not at all). Combine the fractions which behave in the same or similar ways. The first-formed fractions crystallise most readily, the last-formed remain amorphous. Carry the process of separation further for each of the fractions by repeated extraction with methyl alcohol containing

10 per cent of chloroform and with methyl alcohol at 50°. Separate, by filtration, the precipitates produced on cooling, test samples for power to crystallise as before and combine the fractions which exhibit similar powers. As the number of extractions increases the substance becomes progressively poorer in non-crystallising material which occurs in relatively highest concentrations in the first extracts. Finally, pure cerebrone which crystallises completely is obtained.

Cerebrone is a dextrorotatory substance, $[\alpha]_D^{50°}$ for an approximately 5 per cent solution in methyl alcohol containing 75 per cent of chloroform being $+7{\cdot}6°$.

With cerebrone the Molisch carbohydrate reaction (see p. 72) is positive : suspend a trace of cerebrone in 0·5 c.c. of water, by heating for a short time, cool, add one drop of a 15 per cent solution of α-naphthol in methyl alcohol and 1 c.c. of pure concentrated sulphuric acid and shake vigorously. The liquid becomes violet in colour.

Nervone or nervonyl-sphingosine-galactose occurs in brain.

STEROLS

The sterols which frequently accompany the lipins in nature are secondary alcohols of high molecular weight containing several partially hydrogenated ring-systems. Usually they contain from one to three additional double bonds. Saturated sterols, however, are also found in small amount in nature where they usually occur mixed with related unsaturated compounds. According to their place of origin they are termed either zoosterols or phytosterols.

Our knowledge of the physiological significance of the sterols has been increased only to the extent to which they exhibit important structural relationships to the bile acids (see p. 96), the sex hormones and the antirachitic vitamin-*D* (see p. 94). All other questions—for example, those of synthesis, transformation and share in life processes—have not yet been elucidated. Study of constitution, on the other hand, has been in progress for decades and may now be regarded as to some extent complete (Wieland, Windaus [1]).

The presence in the sterols of the free hydroxyl group is the factor which conditions their precipitation with digitonin ; molecular compounds, which crystallise well, being produced. The hydroxyl group is also the point of attack in reactions involving oxidation : the reaction with chromic acid produces first ketones and then, with cleavage of the ring system, dicarboxylic acids. Being unsaturated compounds, sterols can be hydrogenated and halogenated. A method of determination depends on the transfer of oxygen from perbenzoic acid to the double bonds.

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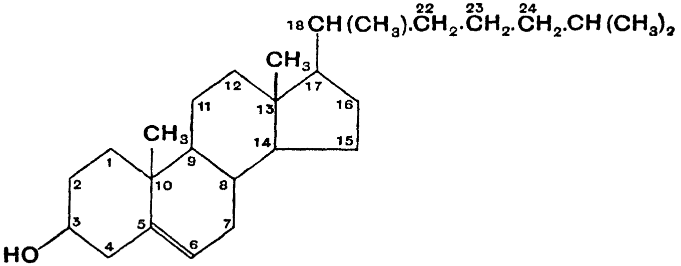
[1] **Wieland and Dane, *Z. physiol. Chem.*, 1932, 212, 41, 263 ; Windaus, *Z. physiol. Chem.*, 1932, 213, 147.**

Sterols take part in some characteristic colour reactions.

1. **The Liebermann-Burchard Reaction.**—Dissolve a little sterol in chloroform and add a few drops of acetic anhydride and of concentrated sulphuric acid. A beautiful, red colour, which changes first to blue and finally to intense green, is produced.

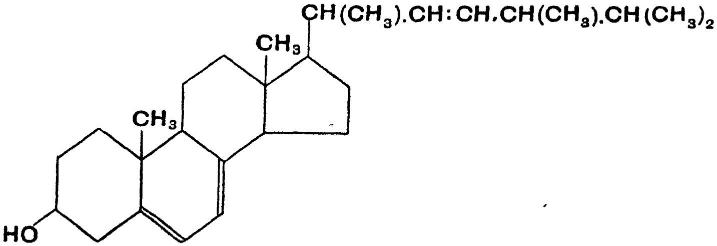
2. **Salkowski's Reaction.**—Add to the solution in chloroform an equal volume of sulphuric acid (*d* 1·76 ; ten parts of sulphuric acid and one part of water). The chloroform acquires a green fluorescence whilst the acid becomes first yellow then red. In the case of ergosterol the red colour only is produced.

Cholesterol, $C_{27}H_{46}O$, melting point 145°, was discovered by Conradi in gall-stones. In the free state or esterified with fatty acids it occurs in most organs and in blood.



Coprosterol, dihydrocholesterol, melting point 101°–102°, is a constituent of faeces.

Ergosterol, $C_{28}H_{44}O$, melting point 160°–161°, was first discovered in ergot and can be detected by means of its characteristic absorption



spectrum. Nowadays it is prepared in important amounts from yeast. It is the precursor of the *antirachitic vitamin* (D_2), melting point 116°, into which it can be converted, by way of a series of isomeric or stereoisomeric intermediate compounds, by irradiation with ultra-violet light :[1]

Ergosterol ⟶ lumisterol ⟶ tachysterol ⟶ vitamin-D_2
⟶ suprasterol.

The last is an " over-irradiation " product and is physiologically inactive.

Preparation of Ergosterol.[2]—In a porcelain dish having a capacity of about 5 litres, grind 2 kg. of pressed yeast with 2 litres of 95 per cent alcohol, transfer the mixture to a 5-litre round-bottomed flask, add a concentrated aqueous solution of 400 g. of potassium hydroxide together with another litre of alcohol and boil the mixture thus obtained for 3 hours on the water-bath. Then filter as dry as possible, with suction, using a large Büchner funnel. Return the insoluble material to the flask, boil with alcohol and filter again. Combine the alcoholic filtrates, concentrate to half volume, add an equal volume of water and shake thoroughly three times in a 5-litre separating funnel with large amounts of ether. Prevent the formation of very troublesome emulsions by filling the funnel to the brim with ether. Finally, wash the golden-yellow ethereal extracts with very dilute hydrochloric acid and dry them with anhydrous sodium sulphate.

Since the insoluble residue on the funnel still contains ergosterol, extract it exhaustively with ether in a Soxhlet apparatus and dry the extract thus obtained with anhydrous sodium sulphate in the same way. Combine the ethereal extracts and remove the ether by distillation from the water-bath. A clear, yellow oil having an odour like that of honey remains. This oil gradually solidifies to a semi-solid crystalline mass. Dissolve this in the smallest possible quantity of low-boiling light petroleum, cool the solution in a freezing mixture, filter off, with suction, the white crystalline precipitate which separates after a short time and wash with cooled light petroleum. The crude product so obtained melts at 141°–142°. Recrystallise it repeatedly from alcohol until the melting point has risen to 154°. The crystals are fine plates having a silvery lustre. They must be protected from light and air as, otherwise, they acquire a yellow colour. The yield from 10 kg. of pressed yeast is about 15 g. of ergosterol.

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[1] Windaus, Linsert, Lüttringhaus and Weidlich, *Annalen*, 1931, 492, 226; Hume, *Nutr. Abs.*, 1936–1937, 6, 891.

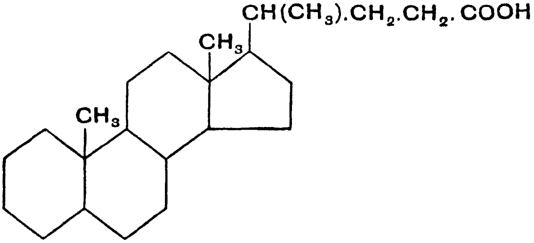
[2] Windaus and Grosskopf, *Z. physiol. Chem.*, 1923, 124, 8.

Stigmasterol, $C_{29}H_{48}O$, melting point 169°–170°, is a constituent of the oil of soya beans.

BILE ACIDS

The bile acids exhibit close constitutional relationships to the sterols. These acids are produced in the liver and stored in the gall-bladder. They are of physiological importance because of their power to facilitate the dissolution in water of a series of otherwise insoluble substances—in particular fatty acids and cholesterol—and so to make possible the absorption of these substances in the organism.

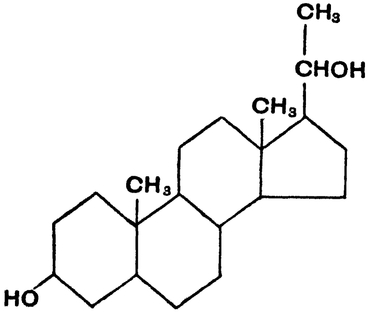
Cholanic Acid may be taken as the prototype of the bile acids.



A whole series of bile acids is derived from this compound by substitution of one or more hydroxyl groups.[1]

SEX HORMONES

The ring skeleton of the sterols is also found in the sex hormones and probably, too, in the toad venoms. In 1929 Marrian [2] isolated from pregnancy urine a substance which proved to be an intermediate in the production of sex hormones but which is biologically inactive because it lacks a CO-group :



Pregnandiol $C_{21}H_{36}O_2$.
Melting point 235°

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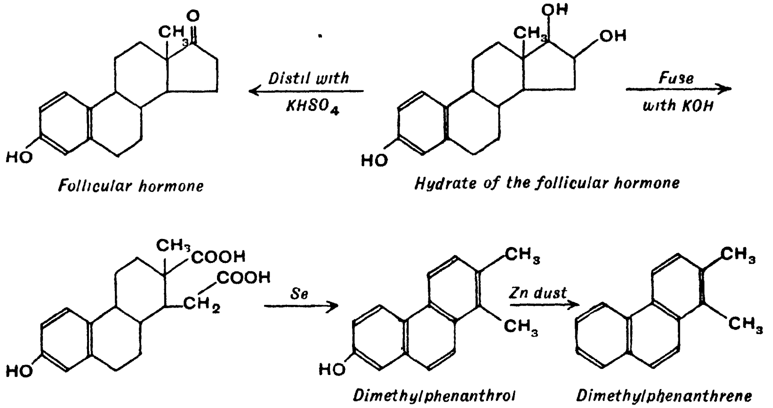
[1] Cf. Dane, *Angew. Chem.*, 1934, 47, 351.

[2] *Biochem. J.*, 1929, 23, 1090.

The hormone which is believed to be responsible for the normal course of the oestrus cycle in the female animal, namely, the *a-follicular hormone* (*a-folliculin*), $C_{18}H_{22}O_2$, melting point 255°, likewise possesses both an OH- and a CO-group. If one or both of these groups is greatly altered the oestrogenic action disappears.[1] Butenandt and his co-workers established the constitution of this hormone in the manner shown in the following scheme, using as starting material the *hydrate of the follicular hormone*, $C_{18}H_{24}O_3$.[2]



By means of the first reaction shown in the scheme, namely, the elimination of water from the hydrate of the follicular hormone by distillation with potassium hydrogen sulphate, Butenandt showed the relationship between this hormone hydrate first isolated by Marrian and the α-follicular hormone. In this way the relationship between the two was proved. On reduction, the CO-group of the α-follicular hormone is converted into a CHOH-group and, at the same time, the activity of the hormone is increased from five- to seven-fold.[3]

Isomerides of the α-follicular hormone are known and are termed the β- and γ- follicular hormones. *Equilin* and *hippulin* $C_{18}H_{20}O_2$, and also *equilenin* $C_{18}H_{18}O_2$, on the other hand, contain less hydrogen than do the follicular hormones.

The preparation of α-folliculin from mares' urine and pregnancy urine[4] and also, as has more recently been found, from the urine of stallions[5] involves the working up of large amounts of material. The urine is made slightly acid and, by a process of extraction with ether or other organic solvent (various solvents such as aqueous alcohol-light petroleum, and aqueous alcohol-benzene are used), there are obtained first a " crude oil " and then an oil so far purified that crops of crude crystalline material can be obtained from it by fractional distillation in a high vacuum.

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[1] For example, the semicarbazone is inactive but the benzoate, because it is slowly hydrolysed in the animal organism, is active. [2] *Ber.*, 1933, 66 [B], 601.

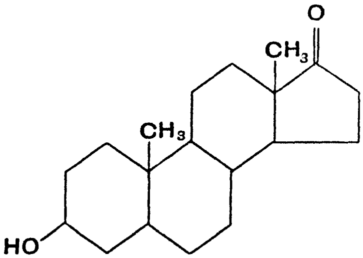
[3] Schoeller, Schwenk and Hildebrandt, *Naturwiss.*, 1933, 21, 286.

[4] Butenandt, *Z. physiol. Chem.*, 1930, 191, 127.

[5] Zondek, *Nature*, 1934, 133, 209.

The glandular transformations which succeed the ripening and bursting of the follicles in the mucous membrane of the uterus result from the action of the *hormone of the corpus luteum.* The " yellow bodies " which are thus produced are also therefore the sole sources so far known of this hormone, which, according to Butenandt and Westphal,[1] probably has the composition $C_{21}H_{30}O_2$, melting point 128·5°. It is a diketone.

The **testicular hormone** (androsterone), $C_{19}H_{30}O_2$, which has been prepared in the crystalline state from male human urine melts at 182°–183°. According to Butenandt[2] and Ruzicka[3] it has the following constitution :



On hydrogenation, androsterone yields dihydroandrosterone which, like dihydrofolliculin has much greater biological activity than has the non-hydrogenated compound. Dehydroandrosterone, on the other hand, which is found together with androsterone itself during the working up of male urine is less active than androsterone.

Ruzicka and his co-workers[4] synthesised androsterone by hydrogenation of cholesterol (see p. 94), epimerisation of the hydroxyl group and degradation of the long side chain.

CAROTENOIDS

The carotenoids are fat-soluble substances which are found widely distributed in the vegetable kingdom.[5] Constitutionally they are closely related to the chief representative of this class of compounds, namely, *carotene.* Carotene exists in various isomeric forms. Some of the carotenoids are hydrocarbons (carotene, lycopene), some are alcohols (xanthophyll, lutein) and some are acids (bixin, crocetin).

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[1] *Z. physiol. Chem.*, 1934, 227, 84.

[2] *Naturwiss.*, 1933, 21, 49.

[3] *Helv. Chim. Acta*, 1934, 17, 1395.

[4] *Loc. cit.*

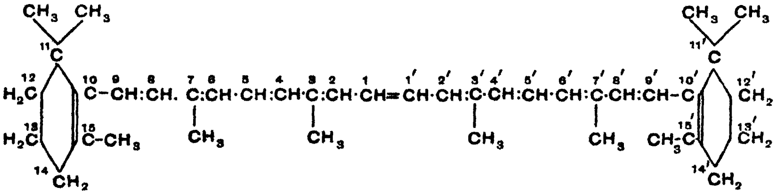
[5] Apparently only plants, higher as well as lower, can synthesise carotenoids. Lederer, *Compt. rend.*, 1933, 197, 1649 ; Chargaff, *Compt. rend.*, 1933, 197, 946.

Carotene is the yellow pigment of carrots. It is also found in paprika, in mountain ash berries, in many green plants such as spinach, in marigolds, in cereals, in tea leaves, in red palm oil, in yolk of egg and so on. *Lycopene,* which is isomeric with carotene, is obtained from tomatoes, rose hips and water-melons : it does not occur in the animal kingdom.

The carotenoids are insoluble in water but soluble in fats (hence the term *lipochrome* which is also applied to them) and in solvents which dissolve fats. The preparation of carotenoids and the separation of the various components of mixtures of carotenoids depends on differing solubilities in alcohol-light petroleum (or benzene)-water mixtures. If an absolute alcoholic extract is treated with light petroleum or benzene the mixture separates into two phases when some water is added. The light petroleum (or benzene) phase contains carotene whilst the alcoholic phase contains xanthophyll, lycopene and the esters of xanthophyll. The separation is carried further and completed by the use of methyl alcohol-benzene mixtures and very frequently by the application of chromatographic adsorption (see p. 40).

The carotenoids are deeply coloured compounds which crystallise well and have sharp melting points. The light absorption bands of their solutions in carbon bisulphide and light petroleum [1] are very characteristic and the halochromic phenomena which they cause with antimony and arsenic trichloride in trichloro-acetic acid or carbon tetrachloride are remarkable.

Constitution.—*β-Carotene,* $C_{40}H_{56}$, melting point 184°, has the following constitution :



In *α-carotene,* melting point 187°, the only constitutional difference is that the double bond in the ring is between C^{14} and C^{15} instead of between C^{10} and C^{15} (the double bond between $C^{10'}$ and $C^{15'}$ remains unaltered [1]). *γ-Carotene*, melting point 178°, can be regarded as derived from β-carotene by the introduction of a double bond between C^{11} and C^{12} and ring cleavage between C^{10} and C^{11}.

---

[1] Winterstein, *Angew. Chem.*, 1934, 47, 315.

Isolation of Carotene from Carrots.[2]—Choose carrots which are as red in the middle as possible and pass them through a mincing machine, taking care not to express too much juice. Dry the minced material at a temperature *not exceeding* 40°–60°, obtaining in this way in the course of 2 or 3 days 1 kg. of dried product from each 10 kg. of fresh carrots. The dried material is no longer sticky, but brittle ; and no longer red, but brown. Grind it up, and, if desired, test it colorimetrically.

For the extraction use 3 litres of light petroleum (b. p. 70°) for each kilogramme of carrots. Extract either in a percolator or by repeated shaking in bottles, leaving to stand for 12 hours until extraction is complete. Combine the filtered extracts, concentrate them to 100–200 c.c. on the water-bath at 30°–40° under reduced pressure and add about 100 c.c. of carbon disulphide. Now precipitate the carotene with absolute alcohol (three to five volumes, total volume, *e.g.* 1 litre) added in small portions at 2–5 minute intervals with shaking. At first only wax is precipitated. Keep a linen filter in readiness but do not filter off the wax until the first beautiful glittering crystals of carotene appear. Then filter rapidly, add the remainder of the alcohol and leave overnight in the refrigerator in an atmosphere of carbon dioxide. Examine under the microscope for the presence of colourless impurities.

Filter with suction, dissolve in a little carbon disulphide, precipitate with alcohol and recrystallise from light petroleum. Preferably, however, extract first with 20–30 c.c. of light petroleum at 50°–60° and reject the extract which contains wax. Then dissolve the pigment in a larger amount of the warm solvent and allow to crystallise in the refrigerator. Dry the preparation in a vacuum desiccator which has first been filled with carbon dioxide and preserve in the dark in a sealed tube containing carbon dioxide.

The yield of carotene from 1 kg. of dried carrots amounts to about 1 g. (about 0·1 g. from 1 kg. of fresh material).

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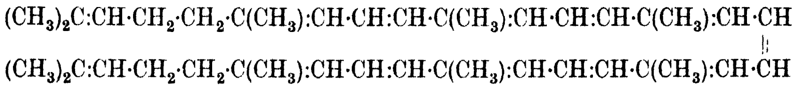
[1] The alteration makes C^{10} asymmetric ; hence α-carotene in contrast to β-carotene is optically active.

[2] Willstätter and Escher, *Z. physiol. Chem.*, 1910, **64**, 47. For the preparation of carotene from paprika see Zechmeister and von Cholnoky, *Annalen*, 1927, **455**, 70.

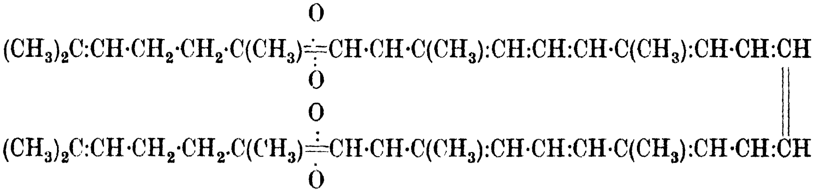
As the example of γ-carotene shows, the aliphatic chain by no means always terminates in rings. *Lycopene,* $C_{40}H_{56}$, melting point 175°, is a carotenoid having a completely open chain.



$$\begin{array}{r}\mathrm{(CH_3)_2C{:}CH{\cdot}CH_2{\cdot}CH_2{\cdot}C(CH_3){:}CH{\cdot}CH{:}CH{\cdot}C(CH_3){:}CH{\cdot}CH{:}CH{\cdot}C(CH_3){:}CH{\cdot}CH}\\ \|\\ \mathrm{(CH_3)_2C{:}CH{\cdot}CH_2{\cdot}CH_2{\cdot}C(CH_3){:}CH{\cdot}CH{:}CH{\cdot}C(CH_3){:}CH{\cdot}CH{:}CH{\cdot}C(CH_3){:}CH{\cdot}CH}\end{array}$$

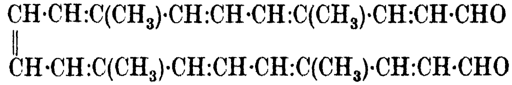
Lycopene

Carotenoids containing oxygen can be obtained, as may readily be supposed, by oxidative degradation of the parent hydrocarbons. Lycopene, for example, when attacked by chromic acid at one end of the chain gives *methylheptenone* $(CH_3)_2C{:}CH{\cdot}CH_2{\cdot}CH_2COCH_3$, and *lycopenal* $C_{32}H_{42}O$:



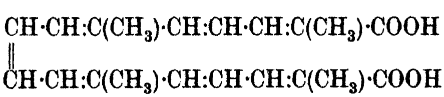
$$\begin{array}{r}\mathrm{(CH_3)_2C{:}CH{\cdot}CH_2{\cdot}CH_2{\cdot}C(CH_3)\overset{O}{\underset{O}{=}}CH{\cdot}CH{\cdot}C(CH_3){:}CH{:}CH{:}CH{\cdot}C(CH_3){:}CH{\cdot}CH{:}CH}\\ \|\\ \mathrm{(CH_3)_2C{:}CH{\cdot}CH_2{\cdot}CH_2.C(CH_3)\overset{O}{\underset{O}{=}}CH{\cdot}CH{\cdot}C(CH_3){:}CH{\cdot}CH{:}CH{\cdot}C(CH_3){:}CH{\cdot}CH{:}CH}\end{array}$$

and when attacked at both ends, 2 moles of methylheptenone and *bixin dialdehyde* :



$$\begin{array}{l}\mathrm{CH{\cdot}CH{:}C(CH_3){\cdot}CH{:}CH{\cdot}CH{:}C(CH_3){\cdot}CH{:}CH{\cdot}CHO}\\ \|\\ \mathrm{CH{\cdot}CH{:}C(CH_3){\cdot}CH{:}CH{\cdot}CH{:}C(CH_3){\cdot}CH{:}CH{\cdot}CHO}\end{array}$$

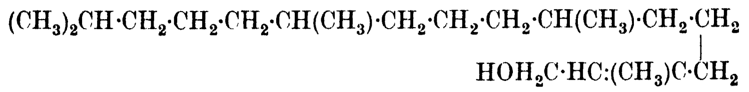
The dicarboxylic acid corresponding to this aldehyde, namely, *norbixin*, $C_{24}H_{48}O_4$, yields *crocetin* when the carbon chain is still further shortened :



$$\begin{array}{l}\mathrm{CH{\cdot}CH{:}C(CH_3){\cdot}CH{:}CH{\cdot}CH{:}C(CH_3){\cdot}COOH}\\ \|\\ \mathrm{CH{\cdot}CH{:}C(CH_3){\cdot}CH{:}CH{\cdot}CH{:}C(CH_3){\cdot}COOH}\end{array}$$

A substance which is very widely distributed in nature and is likewise closely related to the carotenoids is *phytol*, $C_{20}H_{40}O$. Phytol is a monohydric, colourless alcohol which occurs in esterified form as phaeophorbide in chlorophyll.

F. G. Fischer synthesised phytol from ψ-ionone [1] and showed that the alcohol has the following formula :

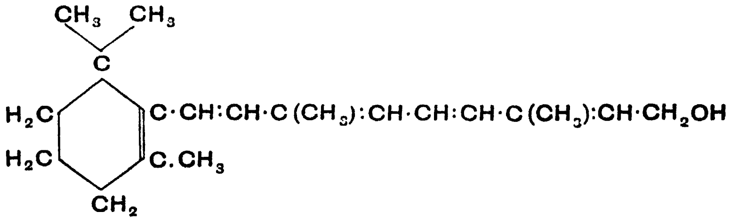


$$\begin{array}{r}(CH_3)_2CH\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(CH_3)\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(CH_3)\cdot CH_2\cdot CH_2\\ | \\ HOH_2C\cdot HC{:}(CH_3)C\cdot CH_2\end{array}$$

For the preparation of phytol from chlorophyll, or from the magnesium-free derivative phäophytin, consult the papers of Willstätter and his co-workers.[2]

VITAMIN-*A*

Between the three carotenes and vitamin-*A*, which occurs as a fat-soluble constituent in butter and also in liver oils, there are interesting connexions. Moore [3] showed that vitamin-*A* is stored in the liver of animals following feeding with carotene. Accordingly, carotene is to be regarded as the biological precursor of vitamin-*A* in the animal organism. Chemical investigation of highly purified vitamin-*A* leads to the following formula from which it is evident that the vitamin may be derived from carotene.[4]



The best starting material for the preparation of highly concentrated material which is capable of being distilled—crystalline material has not yet been obtained—is the liver oil of fish, especially that of the halibut (*Hippoglossus hippoglossus*).

In man, and in the rat, absence of vitamin-*A* from the diet produces opacity and cornification of the cornea (xerophthalmia) and consequently blindness. In addition, loss of weight and finally death supervene in the case of young rats.

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[1] *Annalen*, 1929, **475**, 183.

[2] *Annalen*, 1907, **354**, 205; 1910–1911, **378**, 73.

[3] *Biochem. J.*, 1930, **24**, 692.

[4] Karrer *et al.*, *Helv. Chim. Acta*, 1931, **14**, 1036, 1431. Zechmeister, *Carotinoide* (Berlin, 1934); Lederer, *Les Caroténoides des Plantes* (Paris, 1934). See also Kuhn and Morris, *Ber.*, 1937, **70** [B], 853.

Of great importance as a guide in the study of vitamin-*A* is the *reaction of Carr and Price.*[1] It is carried out as follows: Add to 0·2 c.c. of a 20 per cent solution in chloroform of the material under investigation (*e.g.* liver oil or carotene) 2 c.c. of a saturated solution of antimony trichloride in chloroform. A blue colour, which fades relatively quickly, is produced. This test can be adapted for quantitative determination.

B. ENZYMIC HYDROLYSES

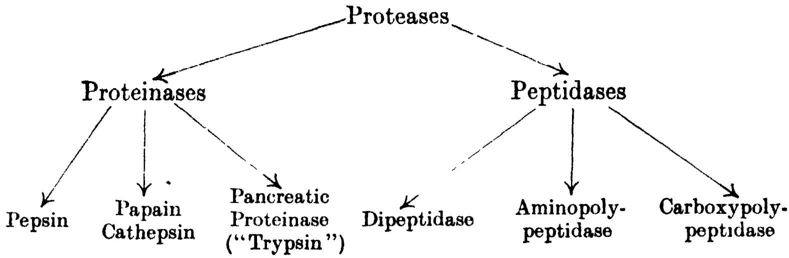
1. PROTEASES

All those enzymes which are concerned in the degradation of proteins to the simplest amino-acids are included in the protease group. Although all these enzymes resemble each other in their behaviour towards proteins they differ from each other sharply in their modes of action and in other ways, and can be separated from each other by appropriate methods of adsorption. All proteolytic enzymes which have been closely studied have been found to consist of components which can be separated from each other—for example, the protease mixture of the pancreas known as trypsin, the enzyme mixtures of the intestinal mucous membrane, those of other organs such as the spleen and kidney and those of lower or higher plants.

In these mixtures of enzymes two main groups of proteases occur in association, namely, the *proteinases* which attack true proteins but not their lower degradation products or such synthetic peptides as have been prepared up to the present; and the *peptidases* which hydrolyse only the intermediate products of protein degradation and the synthetic peptides. The peptidases do not attack proteins. Apparently the proteinases carry the degradation of proteins as far as the middle stages of decomposition, namely, the peptones and peptides, whilst the peptidases complete the degradation by converting the peptones and peptides into amino-acids.

[1] *Biochem. J.*, 1926, 20, 497.

The most important proteases can be classified as shown in the following scheme which takes account of their specificities :



The specificity of the peptidases has been tested with many peptides and to a rather great extent explained.[1] *Dipeptidases* can hydrolyse only dipeptides from natural *l*-amino-acids. *Amino-polypeptidase* can hydrolyse polypeptides composed of three or more amino-acid residues but neither dipeptides nor proteins. The first part of the peptide to be attacked is that containing the amino-group ; if the amino-group is blocked or is part of a non-physiological *d*-amino-acid residue there is no action. *Carboxy-polypeptidase* can be regarded as the " opposite " of aminopoly-peptidase. It attacks polypeptides beginning at the carboxy-group, which must not be altered or blocked. On the other hand, substituents in the amino-group of the substrate do not interfere with the action of carboxypolypeptidase.

Much less precise and more superficial is the classification, on the basis of specificity, of the proteinases because, up to the present, no substrate of well-defined structure, capable of being hydrolysed by proteinases, has yet been discovered. Accordingly, the proteinases must be characterised in other ways, such as by the dependence of their activity on p_H, their behaviour towards activators and so forth. The most recent discoveries, however, seem to indicate that relatively simple synthetic substrates are capable of being attacked by proteinases.[2]

Pepsin, which acts at strongly acid reaction, attacks negatively charged proteins.

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[1] Cf. Grassmann in Oppenheimer's *Die Fermente und ihre Wirkungen*, vol. iii., pp. 99 ff. (Leipzig, 1929) ; Bergmann *et al.*, *e.g. Z. physiol. Chem.*, 1934, **224**, 11, 17, 26, 33, 40, 45, 52 ; Waldschmidt-Leitz, *Enzyme Actions and Properties* (London, 1929) ; J. B. S. Haldane, *Enzymes* (London, 1930).

[2] Shibata, *Acta Phytochim.*, 1935, **8**, 173 ; Tazawa, *Acta Phytochim.*, 1935, **8**, 331.

Pancreatic proteinase acts at alkaline reaction and probably is adapted to attack positively charged protein. In order that it may display its activity to the full this proteinase requires a co-enzyme, namely, enterokinase.

Papain from plants and **cathepsin** from animals occupy an intermediate position for they attack proteins at their iso-electric points at neutral or faintly alkaline reaction. Phyto- or zoo-kinases serve as activators (see p. 116).

In addition to those enzymes of the peptidase and proteinase groups which have been mentioned there are a few enzymes having a higher degree of specificity, as, for example, *prolinase* which liberates the terminal proline residue from peptides containing this amino-acid, and *protaminase* which liberates the arginine at the carboxyl end of the protamine chain.

ACTIVATION OF PANCREATIC PROTEINASE BY ENTEROKINASE

The proteinase of fresh pancreatic extracts has only a slight effect on proteins. The effect can be greatly increased, however, by the addition of enterokinase, a thermo-labile substance which occurs in the intestinal mucous membrane. According to Waldschmidt-Leitz, enterokinase reacts in stoichiometrical proportions with the proteinase, the reaction requiring a measurable time for its completion.[1] Pancreatic extracts which are no longer fresh, however, exhibit not inconsiderable activity even without the addition of the kinase either because enterokinase is spontaneously produced by auto-digestion (Waldschmidt-Leitz) or because inhibitors are destroyed (Dyckerhoff).

Quite fresh pancreatic extracts are used for the preparation of inactive " pancreatic trypsin " (proteinase + carboxypolypeptidase), *i.e.* trypsin which is active only after addition of kinase. The slight activity which such extracts do possess can be suppressed by addition of some caseinate solution and subsequent acidification. The precipitate of casein thus produced in the solution of the enzyme preferentially adsorbs the active proteinase whilst the inactive enzyme remains in the solution.[2]

Enterokinase is best obtained from intestinal mucous membrane which has been dried with acetone-ether mixture. The ereptic components must first be removed. The kinase can be fairly highly purified and its properties (behaviour towards adsorbents) resemble those of the enzymes.

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[1] *Z. physiol. Chem.*, 1924, **132**, 181. Cf., on the other hand, Dyckerhoff, Miehler and Tadsen, *Biochem. Z.*, 1933, **268**, 17.

[2] Waldschmidt-Leitz and Linderstrøm-Lang, *Z. physiol. Chem.*, 1927, **166**, 241.

Preparation of Enterokinase.—Take the small intestines of as many pigs as possible, cut off the upper portion which is about 1 metre long and cautiously scrape out the soft, innermost mucous membrane with a knife or, better, with a glass plate, taking care to keep the material as free as possible from connective tissue and muscle-fibre. Shake the scrapings well with three volumes of acetone and leave for 2 hours. Then filter. Again shake the material from the filter with three volumes of acetone, filter, wash first with 1·5 volumes of acetone and 1·5 volumes of ether, then twice with three volumes of ether (the measurements refer to the original weight of the membrane) and dry on filter paper in the air. The colourless material thus obtained has about one-seventh of the weight of the fresh membrane. Grind to a fine powder in a mill and free from admixed fibrous material by passing through a fine sieve.

The ereptic enzymes of the membrane are removed by treatment with glycerol which dissolves practically none of the kinase. Thereafter the kinase of the dried material (powdered intestine) is extracted with alkaline liquids. For example, shake 4 g. of the powder with 225 c.c. of 87 per cent glycerol, leave for 40 hours at 30° and separate solid from liquid in a centrifuge. Wash the solid with 50 c.c. of 20 per cent glycerol, and leave in contact with 200 c.c. of 0·05 N-ammonia solution at 30° for 3 hours. Filter, and rapidly concentrate the filtrate at 30°–35° in a Faust-Heim apparatus, using a fan to supply the current of warm air until the liquid is no longer alkaline. (In this way the ammonia, which has a detrimental effect, is removed.) Then precipitate the kinase by addition of acetone and ether, and wash successively with acetone and ether which dry the precipitate. Kinase so obtained is free from erepsin and can be preserved for a long time.

The kinase can be very readily adsorbed from acid solution by alumina and removed from it by elution with alkaline phosphate solution containing glycerol as stabilising agent.

METHODS FOR THE DETERMINATION OF PROTEASES

(*a*) **Determination of Tryptic Activity.**[1]—The process depends on the maximal activation of the proteinase by enterokinase (*q.v.*). In this way the variable effects of activators and inhibitors are to a large extent excluded. The activation of trypsin by enterokinase requires time. Accordingly, a period of 30 minutes at 30° is allowed. The substrate used is casein which is rapidly and fairly completely hydrolysed. The p_H is adjusted to the optimal, namely, 8·9, by the use of ammonia-ammonium chloride buffer solution. The increase in the acidity of a 90 per cent alcoholic solution measures the degree of hydrolysis (see p. 58). On each occasion a blank determination modelled exactly on the actual determination must be made and the increase in acidity so determined must be deducted from the result.

Solutions required :

(1) A 0·2 *N*-solution of purest potassium hydroxide in 90 per cent alcohol.

(2) A 0·5 per cent alcoholic solution of thymolphthalein.

Procedure.—Use a 1 : 10 glycerol extract of dried pancreas (see p. 110). Directly before making the determination, dilute the extract ten-fold with water. Pour 1 c.c. of the solution or an equivalent amount of another solution or of a dried preparation and 0·3 c.c. of an enterokinase solution (containing about 1 mg. of the material prepared as described above) into a small flask having a ground-glass stopper, dilute with water to 3 c.c. and leave for 30 minutes at 30°. Then add 5 c.c. of 6 per cent casein solution[2] (Hammarsten) and 2·0 c.c. of N-NH_3-NH_4Cl buffer solution (1 : 1), thus adjusting the p_H to 8·9 at the temperature of the determination (30°). Leave for 20 minutes. Then wash quantitatively into the titration flask (preferably a 250 c.c. conical flask) using a mixture of 5 c.c. of water and 15 c.c. of absolute alcohol for the purpose. Add 2 c.c. of the thymolphthalein solution and titrate with 0·2 *N*-KOH until the first faint blue shade of colour is produced. Then add at once 120 c.c. of boiling absolute alcohol and continue the titration until the first greenish-blue shade appears. It is best to carry out the titration in daylight or in a box having a white lining, using a powerful " daylight " lamp.

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[1] Willstätter, Waldschmidt-Leitz, Dunaiturria and Künster, *Z. physiol. Chem.*, 1926, 161, 190.

[2] Suspend 6 g. of casein in 100 c.c. of water containing 6 c.c. of *N*-ammonia and shake in a machine until dissolution is complete.

The unit for tryptic activity is that amount of enzyme which, under the conditions given, causes hydrolysis corresponding to 1·05 c.c. of $0{\cdot}2N$-KOH. This amount is found in about 0·1 c.c. of a ten-fold diluted glycerol extract of dried pancreas.

(*b*) **Determination of Dipeptidase Activity.**[1]—The hydrolysis of *dl*-leucylglycine can be used for the determination of dipeptidase preparations. As in the method for determining trypsin, the course of hydrolysis in alcoholic solution is measured alkalimetrically.

Solutions required :

(1) $0{\cdot}05N$-KOH in 90 per cent alcohol.

(2) 0·5 per cent solution of thymolphthalein in 90 per cent alcohol.

(3) A solution of leucylglycine prepared thus : In a 250 c.c. measuring flask, shake 9·4 g. of *dl*-leucylglycine with about 180 c.c. of water and add to the suspension thus produced 12·5 c.c. of $2{\cdot}5N$-ammonia solution shaking well and warming gently (50°–60°) until, after some time, dissolution is complete. Now add 41·5 c.c. of $0{\cdot}33M$-KH_2PO_4 (Sørensen) and fill to the mark. Test electrometrically or colorimetrically to make sure that the solution so obtained has $p_H = 7{\cdot}8$. If this is not the case, add, drop by drop, either ammonia or $0{\cdot}33M$-KH_2PO_4 until the required value has been reached.

Procedure.—Pour 3 c.c. of the solution of the substrate ($0{\cdot}006M$-*dl*-leucylglycine) into a 5 c.c. measuring flask previously warmed to 40°, add a suitable amount of the enzyme solution, and fill to the mark. Determine the extent of hydrolysis after 1 hour at 40° by titrating 2 c.c. samples of the mixture in 90 per cent alcohol with $0{\cdot}05N$-KOH *immediately* and *at the end* of the experimental period using thymolphthalein as indicator. To do this, add to the aqueous mixture about five drops of the alcoholic solution of thymolphthalein and titrate until distinct colour-change occurs, then pour in immediately 18 c.c. of absolute alcohol (the blue colour now disappears), continue the titration until the feebly bluish-green shade first appears and take the reading on the burette.

The unit of measurement of dipeptidase action is that amount of enzyme which hydrolyses 50 per cent of the *l*-leucylglycine present in 10 c.c. of the solution in 1 hour at 40°. This amount of *l*-leucylglycine (56·4 mg.) corresponds to 1·2 c.c. of 0·05*N*-KOH required for the increase in acidity in the 2 c.c. sample taken for titration.

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[1] Willstätter and Grassmann, *Z. physiol. Chem.*, 1926, **153**, 250.

(*c*) **Determination of Aminopolypeptidase Action.**[1]—As substrates to be hydrolysed by aminopolypeptidase, polypeptides in which the amino-groups are not substituted may be used. The most convenient for the determination is *dl*-leucylglycylglycine.

The solutions used are the same as those employed in the determination of dipeptidase.

Preparation of the Solution of the Substrate.—Treat 3·06 g. of *dl*-leucylglycylglycine with 4·8 c.c. of 0·25*N*-ammonia solution and 5·0 c.c. of *N*-ammonium chloride solution, add 12·5 c.c. of 0·33*M*-phosphate buffer solution of p_H 7·0 (Sørensen) and make up to 50 c.c.

Procedure.—The procedure closely resembles that described for the determination of dipeptidase. Warm 2 c.c. of the substrate solution in a 5 c.c. measuring flask to 40°, add the solution of the enzyme and fill to the mark. Titrate at once, and again after 1 hour in the manner already described.

The unit of measurement of polypeptidase action is five times that amount of enzyme which hydrolyses in 1 hour at 40°, 50 per cent (*i.e.* 12·25 mg.) of the *l*-leucylglycylglycine present in the 2 c.c. sample. This corresponds to an increase in acidity equivalent to 1 c.c. of 0·05*N*-KOH.

Carboxypolypeptidase action is usually determined with chloracetyl-*l*-tyrosine as substrate [2] at p_H 7·4.

SEPARATION INTO ITS COMPONENTS OF THE MIXTURE OF PROTEASES OF THE PANCREAS

Extracts and secretions of the pancreas contain a mixture of tryptic and ereptic enzymes. In parallel with the differences in the power of these enzymes to attack particular substrates there is difference in specific behaviour towards adsorbents. The ereptic enzymes, which attack the amino-groups of the substrates, exhibit electro-negative acid properties and are preferentially adsorbed at acid reaction on electro-positive basic adsorbents [alumina gel,

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[1] Grassmann and Dyckerhoff, *Z. physiol. Chem.*, 1928, 179, 41.

[2] Cf. Waldschmidt-Leitz and Purr, *Ber.*, 1929, 62 [B], 2222.

$Al(OH)_3$]. Thus, they can be separated from tryptic enzymes which remain in the mother liquors after adsorption.

The ereptic constituents recovered from the adsorbent by elution with alkaline liquids can be further separated into dipeptidase, aminopolypeptidase and prolinase. Up to the present such separation of the last from aminopolypeptidase as has been attained has been distinctly unsatisfactory.[1] By selected methods of adsorption the mixture of tryptic enzymes can be separated into proteinase and protaminase on the one hand and carboxypolypeptidase on the other.

The following scheme shows in summary form the course of adsorptive separation of the proteolytic enzymes of the pancreas.

CRUDE PANCREATIC EXTRACT
(Proteinase, Protaminase, Carboxypolypeptidase, Aminopolypeptidase, Prolinase, Dipeptidase)
Adsorption on alumina C_γ at p_H 4

Solution containing proteinase + protaminase + carboxypolypeptidase
Adsorption on alumina C_γ at p_H 7·0

Solution containing proteinase + protaminase

Adsorbed material. Elution gives carboxypolypeptidase

Adsorbed material. Elution gives aminopolypeptidase + prolinase + dipeptidase
Adsorption on $Fe(OH)_3$ at p_H 4·0

Solution containing aminopolypeptidase + prolinase

Adsorbed material. Elution gives dipeptidase

Preparation of Dry Material from the Pancreas of the Pig.[2]—Clean 500–1000 g. of fresh pig's pancreas and pass it four or five times through a mincing machine. Rub the thin pulp so obtained in a mortar with acetone until about five or six volumes of this liquid have been added, and shake well in a flask the suspension so produced. Leave for 1–2 hours and then filter. Transfer the material from the filter funnel to the flask, shake again with the same volume of acetone and repeat the filtration. Repeat the operation still again but using the same volume of a 1 : 1 mixture of acetone and ether and finally wash twice with ether, using two volumes each time. Grind up the granular powdery mass, spread it in a thin layer between sheets of filter paper and leave to dry in the air. The product thus obtained contains very considerable amounts of fibrous material. Grind as finely as possible in a mill and shake through a fine sieve. Replace in the mill the material which does not pass through the sieve, grind again and once more shake on the sieve. Repeat this procedure several times. The fine powder which passes through the sieve contains most of the substance of the gland. The yield of dry fat-free material amounts to almost 20 per cent of the weight of the purified fresh gland and the yield of fine powder to 67 per cent of the total dry weight of the gland. By means of the procedures described above (pp. 107 *et seq.*) determine the proteinase, aminopolypeptidase and dipeptidase contents of the dry powder.

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[1] Grassmann, von Schoenebeck and Auerbach, *Z. physiol. Chem.*, **1932, 210, 1.**

[2] Willstätter and Waldschmidt-Leitz, *Z. physiol. Chem.*, **1922, 125, 132 ; 1923, 126, 143 ; 1924, 134, 217.**

To prepare extracts pour ten parts of glycerol over one part of the powder and leave for 24 hours with frequent shaking. Then filter.

SEPARATION OF PROTEINASE AND CARBOXYPOLYPEPTIDASE FROM PANCREATIC EXTRACT[1]

Procedure.—Mix 50 c.c. of glycerol extract (87 per cent glycerol) of dried pancreas (1 : 10 extract) with 50 c.c. of water and adjust the p_H of the mixture to about 4·2 by adding 3 c.c. of N-acetic acid-acetate buffer solution (of p_H 3·8). Then add 4 c.c. of a suspension of alumina C_γ containing about 100 mg. of Al_2O_3, shake for a short time and spin in a centrifuge. Draw off the supernatant liquid and add alumina to it as before, repeating the whole operation four times more. The ereptic enzymes are now separated. Neutralise the liquid with N-sodium hydroxide solution and test for the presence of proteinase and the absence of ereptic enzymes (dipeptidase and aminopolypeptidase).

Treat the neutralised solution eleven times in the manner described above with 6 c.c. portions of suspension of alumina C_γ (6 c.c. contain about 150 mg. of Al_2O_3). The solution will contain the proteinase and protaminase free from carboxypolypeptidase. Combine the portions of adsorbent carrying the adsorbed material, wash with 40 c.c. of 20 per cent glycerol and elute with 50 c.c. of 0·04 N-ammonia solution (containing 20 per cent of glycerol). The solution thus obtained still contains a small amount of proteinase together with the carboxypolypeptidase and protaminase. By means of a second elution the proteinase is completely removed but at the expense of the carboxypolypeptidase. For the purpose, treat 40 c.c. of the solution obtained by the first elution with 4 c.c. of the suspension (equivalent to 100 mg. of Al_2O_3) of alumina C_γ at p_H 7·0, remove the adsorbent and repeat the process two more times. Wash the adsorbent then with 20 per cent glycerol and elute with 50 c.c. of 0·04 *N*-ammonia solution (containing 20 per cent of glycerol) as before.

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[1] Waldschmidt-Leitz and Purr, *Ber.*, 1929, 62 [B], 2217.

Determine the proteinase content of the residual solution from which the material has been removed by adsorption and the carboxypolypeptidase content of the material separated by adsorption and elution.

It is also possible to isolate the *protaminase* from the residual solution after removal of the carboxypolypeptidase (see above), since the precipitate produced in that solution by ovalbumin preferentially adsorbs the proteinase.[1] Pure proteinase can be obtained from pancreas by Weil's method.[2]

SEPARATION FROM INTESTINAL MUCOUS MEMBRANE OF AMINOPOLYPEPTIDASE AND DIPEPTIDASE

Aminopolypeptidase and dipeptidase can also be obtained by working up ereptic pancreatic extracts made with 87 per cent glycerol.[3] The ereptic portion of the mixture is separated by repeated adsorption with alumina and subsequent elution with dilute ammonia solution containing glycerol. But, since there is a great predominance of tryptic enzymes in pancreatic powder, the yields of aminopolypeptidase and dipeptidase are relatively small. Accordingly, the description which follows is of the working up of the ereptic mixture from intestinal mucous membrane, this mixture having been shown to be qualitatively identical with that from the pancreas but to contain relatively more dipeptidase and aminopolypeptidase.[4] The polypeptidase can be separated from the dipeptidase by the use of a suspension of iron hydroxide by which the dipeptidase is the more easily adsorbed.

Preparation of an Extract of Intestinal Mucous Membrane.—Grind well in a mortar 100 g. of fresh intestinal mucous membrane from the pig with 500 c.c. of 87 per cent glycerol.[1] The enzyme can be kept for months in this glycerol suspension. For the present purpose dilute 50 c.c. of it with 150 c.c. of water (at the earliest after leaving for 1 day at room temperature) and spin in the centrifuge (4000 revolutions per minute) for 5 minutes. For the first stage of purification treat 100 c.c. of the solution (*i.e.* the glycerol extract clarified with water) with 10 c.c. of 0·1 N-acetic acid. Remove in the centrifuge the precipitate produced. Then take 80 c.c. of the solution, add 3 c.c. of N-acetate buffer solution of p_H 3·8 (p_H of the solution 4·0), cool in ice and adsorb the enzyme on iron hydroxide in suspension,[2] using 10 c.c. (containing the equivalent of 330 mg. of Fe_2O_3) of this. Repeat the adsorption twice, using each time 5 c.c. of the iron hydroxide suspension. Neutralise the residual solution from which the dipeptidase has been completely removed. Remove the enzyme from the iron hydroxide by elution with 25 c.c. of 1 per cent solution (containing 20% of glycerol) of disodium hydrogen phosphate. The dipeptidase, albeit in poor yield, is obtained together with a little polypeptidase by neutralisation with N-acetic acid and filtration.

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[1] Waldschmidt-Leitz and Kofranyi, *Z. physiol. Chem.*, 1933, **222**, 148.

[2] *J. Biol. Chem.*, 1934, **105**, 295.

[3] Waldschmidt-Leitz in Oppenheimer's *Die Fermente und ihre Wirkungen*, vol. iv., p. 1029.

[4] Waldschmidt-Leitz and Schäffner, *Z. physiol. Chem.*, 1926, **151**, 31.

After removing the dipeptidase with iron hydroxide, determine the aminopolypeptidase in the residual solution by the method described on p. 109.

PREPARATION OF DIPEPTIDASE AND AMINOPOLYPEPTIDASE FROM YEAST

Tryptic and ereptic enzymes are found in yeast as well as in the secretions of the pancreas and the intestine. After the yeast cells have been killed, enzymic degradation processes (autolysis) set in, resulting in the gradual dissolution of these enzymes (cf. p. 32). Experience has shown that by suitable alteration of the conditions of autolysis the liberation of the enzymes can be directed into quite definite paths. Accordingly, the isolation of the individual enzyme is possible, for, by directing the autolysis suitably (for example, by choosing a suitable cell poison and suitable acidity), the liberation of one enzyme can be stimulated and that of another retarded or even completely suppressed.

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[1] Cf. footnote 4 on p. 112.

[2] Prepare this adsorbent according to the procedure of Willstätter, Kraut and Fremery, *Ber.*, 1924, 57 [B], 1498.

The enzyme solutions produced at neutral reaction in presence of the ethyl acetate or toluene are suitable for the preparation of rather pure *dipeptidase* ; for the removal of proteinase and polypeptidase they are treated at acid reaction with alumina and then fractionally precipitated with acetone.

Preparation of Dipeptidase from Yeast.[1]—Stir 1 kg. of fresh pressed yeast with a mixture of 70 c.c. of toluene and 15 c.c. of ethyl acetate to make a thin paste, add 1 litre of water and progressively neutralise with ammonia the acid produced, avoiding the addition of any excess of alkali. After 90 minutes remove the enzymically almost inactive liquid in a centrifuge, wash the solid matter with about 2 litres of water and suspend again in water to make a volume of 1 litre with addition of toluene. Then leave to autolyse for 20 hours at p_H 6·4–6·8 and finally remove the residual yeast in the centrifuge.

Take 300 c.c. of the solution, adjust the p_H to approximately 5·0 and add 5 c.c. of N-acetate buffer solution of the same reaction. To the mixture add 20 c.c. of a suspension of alumina C_γ (containing 360 mg. of Al_2O_3). Repeat the adsorption three times, each time with 360 mg. of alumina C_γ which has been previously treated with the first-separated liquid from the yeast.

For this purpose adjust (with acetic acid) the p_H of 1000 c.c. of the liquid to approximately 5·0, add 200 c.c. of 0·5 N-acetate buffer solution having the same reaction, treat with a suspension of 1800 mg. of alumina C_γ and make up the volume to 4000 c.c. Separate the alumina gel quantitatively in a centrifuge, transfer to a 100 c.c. measuring flask and suspend in this volume of liquid. (1 c.c. contains 18 mg. of Al_2O_3.) By means of this pre-treatment the surface of the alumina becomes charged with substances which inhibit the adsorption. This alumina adsorbs dipeptidase much less, whilst the aminopolypeptidase is powerfully adsorbed.

Then mix the residual solution twice in succession with 50 c.c. portions of a suspension of ordinary kaolin (1 c.c. containing 300 mg. of kaolin) not treated with HCl and spin in a centrifuge to obtain a clear liquid.

Now mix 120 c.c. of the solution obtained with 65 c.c. of pure acetone, cooling to about 0°. In order to make the reaction exactly neutral add to the acetone such an amount of alkali—*e.g.* in the form of 0·2 N-alcoholic KOH—that the acetone-water mixture in which the precipitate is produced gives with bromothymol blue the colour characteristic for p_H 7. Separate the precipitate at once. It contains the aminopolypeptidase with a small proportion only of dipeptidase. Add a further 60 c.c. of acetone to the liquid. Collect the new precipitate which is produced and dry it with acetone and ether. This precipitate exhibits powerful dipeptidase action but is free from polypeptidase and proteinase. Dipeptidase is rather sensitive material and hence it is necessary to work quickly, and, especially when precipitating with acetone, to keep the temperature low.

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[1] Grassmann and Klenk, *Z. physiol. Chem.*, 1929, **186**, 38.

Purification and Preparation of Aminopolypeptidase from Yeast.[1]—The liberation of this enzyme during the autolysis of yeast at faintly alkaline reaction proceeds slowly ; but if proteolytic enzymes and especially papain are added, the process is greatly accelerated. At the same time the yield and degree of purity of the enzyme are considerably increased. By using 1 g. of papain for each kilogramme of fresh yeast it is possible to cause the liberation of all the polypeptidase present in 72 hours ; and even after 48 hours the yield is quite satisfactory. The material is further purified by precipitation of the enzyme (together with a nucleoprotein fraction) with acid; removal of the nucleic acid with sodium acetate and ammonium sulphate ; and dialysis.

Procedure.—Stir continuously for about 20 minutes 1900 g. of yeast which has been separated in a centrifuge (dry matter content about 20 per cent) with 150 c.c. of chloroform and mix the material so produced with 950 c.c. of water and some toluene. Now pour in ammonia until the liquid is faintly alkaline (turmeric paper should be just coloured brown), add 1·9 g. of papain and leave to autolyse for 48 hours, continually adding ammonia in order to maintain faintly alkaline reaction (p_H about 7·4). When autolysis is complete remove the residual yeast in the centrifuge.

1. Treat the crude solution with just sufficient 2*N*-acetic acid (400 c.c.) to produce very slight turbidity and slight flocculation. This occurs at p_H 4·5–4·7. Remove the precipitate as rapidly as possible in the centrifuge, wash with 1000 c.c. of water brought to p_H 5·0 by addition of a few drops of ammonia and centrifuge again. Suspend the solid thus separated in the smallest possible amount of water, make just alkaline with ammonia (part of the material precipitated by acetic acid is thus dissolved together with the enzyme) and centrifuge.

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[1] Grassmann, Emden and Schneller, *Biochem. Z.*, 1934, 271, 226.

2. Treat 150 c.c. of the solution with 60 g. of analytically pure sodium acetate, leave overnight and remove in the centrifuge the precipitate [1] produced. Saturate the solution containing the sodium acetate with crystalline ammonium sulphate, leave for some hours and again separate the precipitate in the centrifuge.

3. Pour the solution containing sodium acetate and ammonium sulphate into a fish bladder and dialyse first against running tap-water and then against distilled water just until the solution of the enzyme no longer contains SO_4'' or at most contains only very little SO_4''. (Losses occur if the dialysis is continued longer, because, from a certain point in the dialysis onwards, the enzyme becomes very unstable.) Usually an external sign that the dialysis is at an end is the appearance of turbidity in the solution of the enzyme, fine white flocks being produced. (Usually the dialysis lasts about 24 hours.)

At the various stages during the purification, determine the polypeptidase content and also the dry-matter content of a definite volume of the solution. The polypeptidase value is the number of polypeptidase units in 10 mg.

ACTIVATION OF PAPAIN BY THIOL-COMPOUNDS

The most active form of papain is that which occurs in the sap of the tropical tree *Carica papaya* L. The impure commercial product is obtained by drying the sap in the air. Other higher plants and also, for example, yeast contain a proteinase having similar properties. The animal organism also contains an enzyme—cathepsin—which closely resembles papain. Papain exhibits optimal activity at p_H 5·0–7·0. This evidently harmonises with the iso-electric points of most protein substrates. It has been found that papain and cathepsin can be activated by HCN and also by SH-compounds (hydrogen sulphide, cysteine, glutathione) as well as by various other reducing substances (As_2O_3, active hydrogen, ascorbic acid + $Fe^{\cdot\cdot}$). In the vegetable and animal organism these enzymes are associated with natural activators which are termed phyto- or zoo-kinases. In most cases the kinase is glutathione or a similar SH-compound or possibly also ascorbic acid. The activation of papain probably depends on a hydrolysis or reduction of a disulphide group in the enzyme[1] and/or in the substrate.[2] It is possible that the activator[3] may act by entering into complex combination with traces of poisonous heavy metals but it is to be assumed that this is of rather more secondary importance.

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[1] The precipitate consists for the most part of enzymically inactive nucleic acid or nucleotides.

DETERMINATION OF THE DEGREE OF ACTIVATION OF PAPAIN BY HCN[4] OR GLUTATHIONE[5]

As in the case of enterokinase (see p. 105) the activation of papain by hydrocyanic acid requires time. Accordingly, allow the mixture of enzyme and acid to stand for some time at 40° before the actual hydrolysis.

Solutions required :

(1) Filtered solution of papain.

(2) 3 per cent solution of HCN : neutralise with *N*-HCl (using methyl red as indicator) 10 c.c. of a 6 per cent solution analytically pure KCN. About 8 c.c. of acid are required. Make the volume up to 20 c.c. The solution cannot be kept for long.

(3) 0·2*N*-solution of disodium citrate (Sørensen).

(4) 6·66 per cent solution of best commercial gelatin.

(5) A solution containing 200 mg. of reduced glutathione per 10 c.c.

Procedure.—Mix in a 50 c.c. measuring flask 5 c.c. of the disodium citrate solution and 2 c.c. of hydrocyanic acid solution (equivalent to 60 mg. of KCN or 25 mg. of HCN), pour in a suitable amount (from 5 to 40 units) of the solution of the enzyme under examination and make up the volume to 20 c.c. Before adding the enzyme solution adjust its p_H to about 5·0 if necessary. Leave the mixture for 2 hours at 40°, add 30 c.c. (equivalent to 2 g. of gelatin) of the gelatin solution, make up to the mark, shake *very vigorously* for about 20 seconds, at once withdraw a 10 c.c. sample and titrate in alcoholic solution with 0·2*N*-alcoholic solution of KOH, using thymolphthalein as indicator (method of Willstätter and Waldschmidt-Leitz, see p. 107).

Keep the rest of the mixture in a thermostat at 40°. After 1 hour, titrate a second 10 c.c. sample. The difference between the titration values is a measure of the amount of hydrolysis which has occurred. From the curve (Fig. 18) take the number of enzyme units corresponding to the amount of hydrolysis found. The values in the figure refer to a 10 c.c. sample taken for titration and accordingly the amount of enzyme in the whole solution (50 c.c.) is calculated by multiplying by five the number of units taken from the curve.

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[1] Bersin and Logemann, *Z. physiol. Chem.*, **1933**, **220**, 209 ; Bersin, *Z. physiol. Chem.*, 1933, **222**, 177.

[2] Maschmann and Helmert, *Z. physiol. Chem.*, 1934, **223**, 127.

[3] Krebs, *Biochem. Z.*, 1930, **220**, 289.

[4] Willstätter and Grassmann, *Z. physiol. Chem.*, 1924, **138**, 184.

[5] Grassmann, von Schoenebeck and Eibeler, *Z. physiol. Chem.*, 1931, **194**, 124.

If small amounts only of enzyme are available, work preferably on the half-micro-scale, using a solution having one-tenth of the volume given above (*i.e.* total volume 5 c.c.) and titrating 2 c.c. samples with 0·05*N*-KOH. Adjust the calculation accordingly.

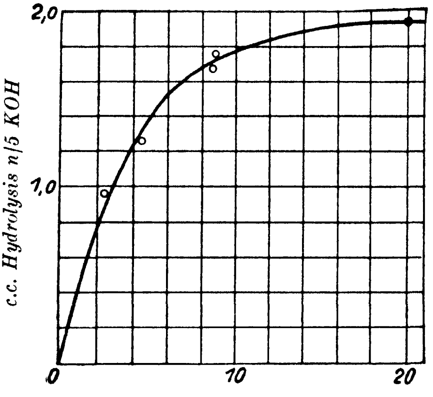

FIG. 18

Activation with Glutathione.—Use a solution having exactly the same composition as that employed for hydrocyanic acid activation but use 2 c.c. of glutathione solution (reduced) instead of the hydrocyanic acid solution. It is not necessary to allow time for incubation.

Preparation and Purification of Glutathione.[1]—Mix well 10 kg. of brewers' yeast and 600 c.c. of ethyl acetate. If the weather is cold, warm the ester to 30° in order to accelerate the autolysis. When the yeast has completely " liquefied " after frequent stirring leave for 20 minutes and then add about an equal volume of water. The yield is not appreciably increased by this dilution but it facilitates rapid deposition of the yeast cells. After a further 20-minute period remove the liquid in a separator.

To the solution, which is still slightly turbid, add saturated lead acetate solution until it ceases to produce a precipitate (about 400 g. of lead acetate are required). The supernatant liquid which has p_H about 5·5 still contains some glutathione. Without first centrifuging, adjust the p_H to 7·0 (bromothymol blue) with concentrated ammonia solution and complete the precipitation with lead acetate. Although the amount of material thus thrown down is small in comparison with the first precipitate, that material contains about 15–20 per cent of the total yield of glutathione. Carefully avoid the use of the slightest excess of lead acetate because the lead salt of glutathione is soluble in excesss. Test the completeness of the precipitation by centrifuging samples occasionally. Leave overnight and decant the liquid or remove it in a separator, make a fine suspension of the lead salt in about 400 c.c. of distilled water and decompose by stirring in vigorously approximately 25 per cent sulphuric acid until the solution is just acid to Congo red. Now leave for some time (20–30 minutes) with frequent stirring. (At the end of this time the solution should still be acid to Congo red.) Then add concentrated sulphuric acid (12 c.c. for each litre of solution) diluted with an equal volume of water in order to make the normality of the acid 0·5. Remove the lead sulphate in the centrifuge and wash with 100 c.c. of 0·5 *N*-sulphuric acid. The whole solution is now 0·5 *N* with respect to sulphuric acid which is important for the isolation of the glutathione by way of the characteristic copper salt. If no precipitation takes place titrate the solution, using Congo red as indicator, in order to add the amount of sulphuric acid which is lacking.

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[1] Procedure of Hopkins, *Biochem. J.*, 1921, **15**, 286; *J. Biol. Chem.*, 1929, **84**, 269, taking advantage of the results of Kendall *et al.*, *J. Biol. Chem.*, 1929, **84**, 657.

The copper salt of glutathione is precipitated by gradual addition, with good stirring, of cuprous oxide. Prepare the oxide as follows: Add glucose solution to a solution containing, in 1 litre, 34·6 g. of copper sulphate, 173 g. of sodium potassium tartrate and 51·6 g. of sodium hydroxide and warm on the water-bath. When the cuprous oxide has settled, decant the liquid, filter with suction, wash and dry.

Treat the solution of glutathione in 0·5 *N*-sulphuric acid with cuprous oxide (dry, or suspended in a little water). The first portions dissolve without producing any precipitate but after some time the copper salt of glutathione separates as a silky, shining precipitate. (The precipitate is occasionally flocculent.) About 1·2 g. of cuprous oxide are required. Again determine the end-point of the precipitation by occasionally removing samples in the centrifuge. Avoid the use of an excess of cuprous oxide. Now leave overnight, draw off the supernatant liquid, centrifuge, wash four or five times with distilled water and use the salt, preferably without drying, for the further operations. The copper salt obstinately retains sulphuric acid (combined, no doubt, with the amino-groups) and gives it up only gradually. The acid is far from completely removed even by washing ten times. Accordingly, the salt cannot safely be dried. But if it is nevertheless desired to do so for the purpose of determining the yield, add to the last wash-water some drops of sodium acetate solution until the filtrate is no longer acid to Congo red. However, the copper salt settles with much more difficulty from the neutralised than from the acid solution on centrifuging.

Actually, the presence of small amounts of sulphuric acid, such as those which remain in the copper salt after four or five washings, is desired for the decomposition of the salt with hydrogen sulphide since they facilitate the precipitation of the copper sulphide.

Suspend the copper salt in ten to twenty parts of water, pass in hydrogen sulphide to saturation and centrifuge until clear. Wash the precipitate of sulphide twice with water containing hydrogen sulphide. Then concentrate the solution to about 50 c.c. under reduced pressure (12 mm.) at a temperature not exceeding 35°, using a capillary tube through which carbon dioxide is drawn. Now precipitate the sulphuric acid quantitatively with barium hydroxide solution, centrifuge and concentrate again to about 25 c.c., using a capillary tube and carbon dioxide as before. Pour the solution into a small beaker, washing out the flask with 90 per cent alcohol and add absolute alcohol until a permanent turbidity is just produced. Place the liquid in a desiccator containing fresh sulphuric acid and a dish of potassium hydroxide ; displace the air by passing in carbon dioxide and cautiously evacuate until bubbles rise in the solution (avoid delayed boiling !). Later, evacuate still further. The glutathione crystallises overnight. Concentrate the mother liquor further in the desiccator until no more crystallisation occurs. Precipitate the residual glutathione in the amorphous state from the residual syrup by adding absolute alcohol. The precipitate thus obtained weighs about 0·2 g.

Filter the crystals with suction, wash carefully six to ten times with 65 per cent alcohol, then successively with small quantities of 70, 80, 90 and 100 per cent alcohol and, finally, with absolute ether. The yield amounts to 2·0–2·5 g. Melting point 192°–193°.

Recrystallisation.—Powder the crude product finely, cover with about an equal volume of boiling water and bring into solution by adding water, drop by drop with stirring. Filter with suction, using a small filter tube, replace the air above the filtrate with carbon dioxide and close tightly. Isolate as described above. The melting point is now about 197° and rises to almost 200° after one or two further recrystallisations. The yield each time is about 50–60 per cent. When the mother liquors are concentrated in a current of carbon dioxide small additional amounts having the same degree of purity are obtained.

2. CARBOHYDRASES

The collective term carbohydrase is applied to all enzymes which can hydrolyse the bond between two simpler sugars in di- and poly-saccharides or the corresponding bond between sugar and aglucone in glucosides.

The carbohydrases are subdivided, just as are the proteases, thus into:

1. Enzymes which attack substances of high molecular weight, such as starch, glycogen, cellulose—namely, the *polysaccharases* or *polyases*. The most important members of this group are animal and vegetable amylase, cellulase and the hemicellulases.
2. Enzymes which attack sugars or glucosides of low molecular weight—namely, the *oligosaccharases* and *glucosidases*.

The investigations of E. Fischer, Willstätter, Kuhn, Weidenhagen and Helferich have shown that the determining factors in the enzymic hydrolysis of polysaccharides and glucosides are the constitutions and configurations of those sugars the *reducing groups* of which constitute the glucosidic link with the rest of the molecule. The nature of this residual part affects only the rate of the degradation. This applies in the first instance only to enzymes which attack the lower carbohydrates. The specificity of the enzymes which attack the carbohydrates of high molecular weight has not yet been elucidated in detail.

The most important specific enzymes from the most extensively studied of the glucosidases are:

(*a*) The *α-glucosidases*, such as the α-glucosidase (maltase) of yeast.

(*b*) *β-Glucosidase*, in particular the β-glucosidase of the emulsin of bitter almonds.

(*c*) β-h-*Fructosidase*, which is known also as "invertase" or yeast saccharase.

There exists, in addition, a whole series of glucosidases which act specifically in the hydrolysis of other sugars (*e.g.* galactose, pentoses) of the glucoside type.

From the above it is evident that an enzyme may break the glucoside links in several different sugars if these links are of the same kind. Thus, for example, the following enzymes hydrolyse the substrates shown :

Enzyme	*Substrates*
α-Glucosidase . .	α-Glucosides, maltose (α-glucosido-glucose), sucrose (α-glucosido-β-*h*-fructose), melezitose (α-glucosido-fructosido-glucoside).
β-Glucosidase . .	β-Glucosides, gentiobiose (β-glucosido-glucose)
β-*h*-Fructosidase .	β-*h*-Methylfructoside, sucrose, raffinose (β-fructosido-glucosido-glucoside).[1]

PREPARATION AND PURIFICATION OF YEAST INVERTASE

Yeast saccharase or invertase which causes the hydrolysis of cane-sugar (sucrose) into equimolecular amounts of glucose and fructose can be obtained from yeast in a relatively high state of purity by the method of Willstätter and his co-workers. Invertase is one of the enzymes with which these investigators carried out their classical experiments on the purification of this class of substances. In the course of these investigations the methods of purification were altered in the most varied way. By the procedure about to be described, invertase can be rapidly prepared in large yield and with a high degree of purity. The starting material is not ordinary yeast but yeast having its invertase content increased about twenty-fold by means of a special fermentation process in which the concentration of sugar is minimal.

In order to liberate the invertase which is anchored to the yeast cells these must be destroyed by addition of toluene. The autolysis which then sets in leads to rapid enzymic degradation of the cellular material with consequent dissolution of the invertase. The acid which is produced during the autolysis is continuously neutralised by addition of ammonium sulphate. In the first few hours of the autolysis small amounts only of invertase are liberated but the accompanying material goes into solution to a great extent during this period. Accordingly, after 3 hours a preliminary separation is made and the autolysis is then allowed to continue for 1 day longer. During this period at least 80 per cent of the invertase is brought into solution.

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[1] Cf. Helferich, *Ergebn. Enzymforsch.*, 1933, 2 74 ; and Weidenhagen, *Ergebn. Enzymforsch.*, 1932, 1, 168.

Concentration of Invertase in Yeast.[1]—On the day before the preparation of invertase is begun, pour 4 litres of nutrient salt solution into a 10-litre filter jar, warm to 28° and add 200 g. of fresh pressed yeast. (The salt solution contains 8 g. of potassium dihydrogen phosphate, 8 g. of ammonium dihydrogen phosphate, 2 g. of potassium nitrate and 2 g. of hydrated magnesium sulphate.) Place the jar in an electrically heated bath in which the temperature is maintained at 27° during the whole course of the experiment and stir the mixture vigorously with a mechanical stirrer. Run in, drop by drop, from a funnel having a capillary stem, 20 per cent cane-sugar solution, at the rate of 100 c.c. per hour (10 per cent of sugar with respect to the weight of pressed yeast). After 2–3 hours separate the yeast from the alcohol-containing solution and renew the nutrient solution. At this stage the separation can easily be accomplished by decantation ; thereafter separate in a centrifuge or, better, in a separator. For convenience allow 50–80 per cent of the sugar to run in during 5–8 hours. Then attach a finer capillary to the stem of the funnel and allow further 10–20 per cent to run in during the night, making the total period about 17 hours. (If the period is somewhat longer the yield is still better.) Next morning separate the yeast, now rich in invertase, in a centrifuge.

Set aside small samples of the original and the enriched yeast for comparison of their invertase contents.

Weidenhagen has recently stated [2] that if air is blown into the liquid during the operation and the concentration of sugar is kept as low as possible, the degree of enrichment is great. In this way the whole process can thus be limited to an 8–10 hour period and decantation is unnecessary.

Preparation of Invertase.—In a 2-litre bottle stir about 250 g. of the enriched yeast with 30 c.c. of toluene and 25 g. of finely powdered diammonium hydrogen phosphate and leave the mixture to autolyse for 1 hour in the thermostat at 30° with occasional stirring. Then add an equal weight of water (*i.e.* equal to the weight of the yeast) and replace in the thermostat for 2 hours with occasional shaking. Centrifuge the mixture, treat the residual yeast with the same volume of water and some toluene and leave for 1 day.

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[1] Willstätter, Lowry and Schneider, *Z. physiol. Chem.*, 1925, **146**, 158.

[2] *Angew. Chem.*, 1934, **47**, 581.

Meanwhile determine, according to the procedure described below, the invertase content of the fresh and the enriched yeast and of the autolysate just separated.

(*a*) In a measuring cylinder shake vigorously 5 g. of the original yeast with water, to make a volume of 50 c.c.; withdraw 20 c.c. of the liquid for determination and complete the determination within 20 minutes. During this period shake several times in order to prevent the yeast from settling.

(*b*) Carry out the same determination with about 0·25 g. of the enriched yeast.

(*c*) In the same way, make a determination with 20 c.c. of the autolysate.

In the first two determinations (*a* and *b*) a few drops of toluene must be added to kill the yeast.

In order to free the invertase solution from protein, cool 300 c.c. of the fresh autolysate to 0° and adjust the p_H to about 5·0 by adding 1 c.c. of *N*-acetic acid. Then mix with an equal volume of alcohol which has been cooled to $-20°$. A small precipitate containing the enzyme is produced. Filter with suction, using two funnels in which the paper is covered with a layer of kieselguhr 1 mm. thick. This layer is easily removed from the paper. Stir the kieselguhr with about 80 c.c. of water. This produces a clear solution of the enzyme which can be filtered easily.

Purify further by adsorbing on alumina. First carry out preliminary tests in order to find out how much alumina suffices to adsorb 80–90 per cent of the invertase present. Where possible arrange the tests so that they include experiments in which about 20, 50 and 80 per cent adsorption occurs. For this purpose, add to equal amounts of the enzyme solution prepared as above, varying amounts of a suspension of alumina (the smallest amount of the suspension should contain about 10 mg. of Al_2O_3), make up to 100 c.c. in a measuring cylinder and shake thoroughly. (Before adding the suspension, shake it for 15 minutes on a machine with glass beads in order to break up any small lumps.) Then centrifuge and determine the amount of residual enzyme in the solution (10 c.c. of solution; stop after 10 minutes).

Having determined the amount of alumina required, adsorb the enzyme from the main solution, and elute at once by suspending in 1 per cent solution of diammonium phosphate (100 c.c. of the solution for each 0·1 g. of alumina). Leave for 30–60 minutes, centrifuge and determine the invertase contents of the main solution and of the liquid with which the elution was carried out.

In order to determine the degree of purity attained (the residue in the phosphate-free solution must be determined), remove the phosphate by dialysis in a fish bladder. Then determine the amount of dry matter in a sample of the solution, and, where possible, determine the invertase content also.

Determination of Invertase Activity.[1]—Determine the time required for a given amount of invertase to hydrolyse 4 g. of cane-sugar in 25 c.c. of solution at 15·5° until the optical rotation is 0°. For this purpose prepare a 32 per cent solution of sugar—test the purity of the sugar by determining its specific rotation—which can be kept for 2 or 3 days only, free from mould. Take 50 c.c. of this solution, add 5 c.c. of a 10 per cent solution of sodium dihydrogen phosphate and transfer to a 100 c.c. flask. When the activity of yeast is being determined or when the experiment lasts for a long time in presence of yeast extract which readily decomposes, it is necessary to add two to five drops of toluene—but not otherwise. Suspend the flask in a thermostat and maintain the temperature at 15·5° ± 0·03° by means of circulating water and a thermo-regulator. (The flask attains this temperature in about 5 minutes.) Add the enzyme from a pipette to the sugar solution noting exactly to 0·1 minute the time when the pipette is half empty. Thereafter mix the solutions well by shaking. Make the liquid now up to the mark with water from a wash bottle which is kept always in the thermostat and finally mix well by shaking. Choose an amount of enzyme such that the inversion of the substrate reaches the stage marked by 0° rotation (75·93 per cent hydrolysis) in 60–180 minutes.

After about 15 minutes take the first sample. Withdraw with a pipette 25 c.c. of the solution and run into a large test-tube containing 5 c.c. of $2N$-sodium carbonate solution. (The carbonate arrests the action of the enzyme and greatly accelerates the mutarotation of the products of hydrolysis, namely, α-glucose and β-fructose.) Again note exactly the time when the pipette has delivered half of its contents; and when it has delivered them all, mix the liquids well by vigorous shaking. The approximate rate of hydrolysis can be calculated by polarising at once and taking two further samples when the degree of hydrolysis is about 40 per cent and 70 per cent respectively. In this way the time required to reach 0° rotation can be calculated exactly. If it is desired to make exact measurements the experiment can be carried out on a larger scale (the volumes of the solutions may be doubled) and more samples may be taken for determination. The final polarisation is made in a 2 dm. tube at 20°, 20–30 minutes after the last sample has been taken.

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[1] Cf. Schneider in Oppenheimer's *Die Fermente und ihre Wirkungen*, vol. iii., p. 765.

The percentage p of sugar inverted is calculated from the angle of rotation d of the samples taken by means of the equation:

$$p = \frac{R - d}{R - L} \times 100,$$

where R and L are the maximal dextro- and laevo-rotations respectively. When the conditions chosen are those described above (4 g. of sugar in 25 c.c.) R, the initial rotation, is 17·72°, and $R - L$, the total decrease in rotation when inversion is complete, is 23·37°. Accordingly:

$$p = \frac{17{\cdot}72 - d}{23{\cdot}37} \times 100.$$

The time t_z for 75·95 per cent hydrolysis (rotation 0°) is calculated from the time τ from the beginning of the experiment until the sample is taken, an experimentally determined time-hydrolysis curve being used. This curve is shown in Fig. 19 (the continuous line); the ordinates represent percentages of sugar hydrolysed, whilst the abscissae represent time T measured in arbitrarily chosen units. T is the time factor in the hydrolysis of the sugar and for the point t_z, when the rotation is 0°, is 13·55. Accordingly:

$$t_z = \frac{\tau}{T} \times 13{\cdot}55.$$

Since the curve of the kinetic process is very markedly asymptotic towards the end of its course it is obvious that extrapolation beyond $p = 80$ per cent is inadmissible. But values from about 25 per cent to, at the most, 83 per cent hydrolysis are suitable for the determination. If samples are taken within this range the values for t_z agree to within ±1 per cent.

In determinations made for preparative purposes, and hence less important, it may suffice to take a single sample only for the determination of t_z. In all other cases several samples must be taken.

When 0·05 g. of material in 25 c.c. of solution is taken for the determination, the time required to hydrolyse the sugar to such an extent that the rotation in Na-light is 0°, after the muta-rotation has been suppressed with sodium carbonate, is the *time factor* of the enzymic material used. If the dry weight of the invertase used to hydrolyse 25 c.c. of the solution of the substrate—$4x$ grammes are pipetted into a 100 c.c. flask—be x grammes, 0° rotation is attained in t_z minutes. Hence 0·05 g. of the enzymic material will carry the hydrolysis of this amount of cane-sugar (4 g.) to the stage where the rotation is 0° in $t_0 = \frac{t_z \times x}{0{\cdot}05}$ minutes. The time factor of the enzymic material is $t_0 = \frac{t_z \times x}{0{\cdot}05}$ minutes. The invertase value is $\frac{0{\cdot}05}{t_z \times x}$; it gives the number of invertase units in 50 mg. of the dry material.

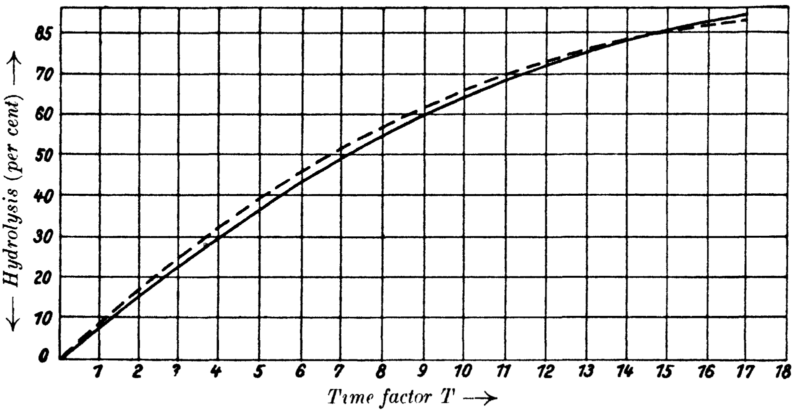

FIG. 19.—Time-hydrolysis curve in hydrolysis by invertase.
- - - - - - - logarithmic curve.
———— curve determined experimentally at 30°.

KINETICS OF ENZYME ACTION. AFFINITY CONSTANTS

It might seem natural to assume for a simple enzymic decomposition, proceeding according to the equation $S \longrightarrow X + Y$[1]

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[1] No account will be taken here of the reversibility of this decomposition ($X + Y \longrightarrow S$; enzymic synthesis). In a few enzymic reactions only, *in vitro, e.g.* hydrolysis of esters, is this reverse process of practical importance.

(*e.g.* the decomposition of cane-sugar into glucose and fructose or of a dipeptide into two moles of amino-acid), that, normally, the reaction would be unimolecular. This is not the case, however, as will be particularly evident from the classical considerations and calculations of Michaelis and his co-workers.[1] These considerations will be discussed here very briefly only. According to the fundamental hypothesis made by Michaelis and abundantly confirmed, the enzyme E and the substrate S unite practically instantaneously to form an unstable intermediate compound ES and the rate of hydrolysis actually observed is dependent, on the one hand, on the prevailing concentration of this complex, and, on the other, on the rate at which it decomposes into the reaction products X and Y with re-formation of the enzyme. Now the concentration of SE is determined by the equilibrium :

$$S_f + E_f \rightleftharpoons [ES] \quad . \quad . \quad . \quad . \quad (1a)$$

or

$$\frac{[S_f]\,[E_f]}{[ES]} = \frac{[S_f]\,[E_f]}{[E_b]} = K . \quad .. \quad . \quad . \quad (1b)$$

Here $[E]$ denotes the *total* concentration of the enzyme, $[E_b]$ the concentration of enzyme *bound* to the substrate and E_f the concentration of the *free* enzyme, whilst $[S_f]$ denotes the *free* portion of the substrate not bound to enzyme. K is the dissociation constant. Its reciprocal is a measure of the affinity, A, of the enzyme for the substrate. The total concentration $[E]$ of the enzyme present is related to the concentration of free enzyme $[E_f]$ thus

$$[E_f] = [E] - [ES] \quad . \quad . \quad . \quad . \quad (2)$$

Since the concentration of the enzyme and accordingly also $[ES]$ can be neglected in all cases when compared with the concentration of the substrate it may be said that

$$[S_f] = [S] \quad . \quad . \quad . \quad . \quad . \quad (3)$$

All substances which combine with free enzyme to produce complexes other than ES and, hence, which remove a part of the enzyme from the system in equilibrium have an inhibitory action on this process. (Other substances also have such action.) Substances of this kind are, in general, the products of hydrolysis, and the final products of the decomposition which still retain in whole or in part the groupings which are important for the combination of the enzyme with the substrate and hence have affinity for the enzyme. Thus we have still the equilibria

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[1] *Biochem. Z.*, **1913, 49, 333.**

$$S_f + X \longrightarrow XS \qquad\qquad S_f + Y \longrightarrow YS$$

$$\frac{[S_f][X]}{[XS]} = K_x \qquad\qquad \frac{[S_f][Y]}{[SY]} = K_y.$$

From what has been said, the following limiting cases can easily be understood on the basis of purely qualitative considerations :

1. If S is *large* and K *small* (enzyme having high affinity for the substrate) the affinity of the enzyme for the products of hydrolysis X and Y may be neglected (K_x and K_y are large or ∞). In this case, until a very high degree of hydrolysis has been attained, the whole of the enzyme is bound to the substrate, $[SE] = [E] =$ a constant. The rate of hydrolysis is constant (the course of the reaction is linear ; a reaction of null order).

2. Conversely, if the affinity for the substrate is small but that for the products of hydrolysis great ($K \gg K_x$; $K \gg K_y$), then the inhibitive action of the reaction products on the course of the hydrolysis will soon cause great reduction in the reaction velocity (great reduction in the constants of reaction of the first order). In practice, the course of the reaction lies between these two limiting cases.

The value of K can be experimentally determined by measuring the initial rate of reaction for a given enzyme-substrate system in which the concentration of enzyme is kept constant and that of the substrate is varied (where necessary the value must be extrapolated to time 0). For at the beginning of the reaction the effect of the reaction products need not be considered since it cannot be experimentally detected. Accordingly

$$\frac{[S_f] \times [E_f]}{[ES]} = K, \qquad . \quad . \quad . \quad . \quad . \quad (1b)$$

which gives the equation

$$\frac{[ES]}{[E]} = \frac{[S]}{K + [S]},$$

when rewritten taking account of equations (2) and (3). Since the concentration $[ES]$ is proportional to the velocity of reaction we can write

$$\frac{v}{v_{\text{max.}}} = \frac{[S]}{K + [S]},$$

where v is the observed velocity corresponding to the concentration $[S]$ and v_{max} the velocity when the enzyme is all bound to the substrate. The following curves show the graphical expression of this equation which is formally the same as that for the residual dissociation curve of an acid

$$a=\frac{K+[H^{\cdot}]}{[H^{\cdot}]}.$$

These curves show the relation between the rate of hydrolysis and the logarithm of the reciprocal of the concentration of the substrate (p_s) for a substrate with high and one with low affinity for the enzyme.

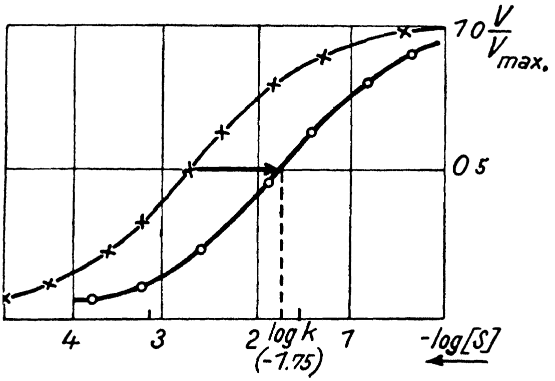

FIG. 20.—Activity-concentration curve for invertase according to Michaelis and Menten, *loc. cit.*

$$\text{For } \frac{v}{v_{\text{max.}}}=0{\cdot}5 \text{ (half of the maximal rate of hydrolysis)}$$

$$K=[S],\ \log K=\log [S],$$

as may easily be shown.

Curves of this kind have been constructed on the basis of experimental results and found to agree excellently with the theory. But that does not mean, of course, that the assumption underlying the view of Michaelis—namely, that in homogenous media there is a stoichiometrical equilibrium reaction between enzyme and substrate—is correct. Formally similar conclusions are reached when it is assumed that there is an adsorption equilibrium between enzyme and substrate and the considerations are based on Langmuir's view about the adsorption isotherm (adsorption in a monomolecular layer).[1]

The theory of Michaelis explains satisfactorily a series of otherwise obscure phenomena. In particular it has acquired importance in the understanding of specificity. When, for example, one substrate S_1 is degraded by a particular enzymic material twice and by another five times as rapidly as is another substrate S_2 it by no means follows that two different enzymes present in varying proportions are to be held responsible for the degradation of the two substrates. Such shifts in the equilibrium quotients may very well be due to differences in the affinities of the two substrates for the enzyme. Thus, to take one factor only, the hydrolysis of that substrate which has the smaller affinity for the enzyme is more powerfully affected by the presence of inhibitors than is the hydrolysis of the substrate having the greater affinity.

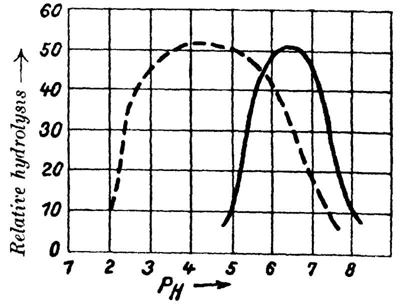

FIG. 21

——— α-Glucosidase
------ β-*h*-Fructosidase

DEPENDENCE OF THE ACTIONS OF MALTASE AND INVERTASE ON p_H

Since the specificity of the enzymes which hydrolyse sugars of low molecular weight concerns only the sugar residue bound by a glucosidic link, sucrose, in which both hexoses are united by such links, must be hydrolysed by two enzymes. On the one hand α-glucosidase must split off α-glucose and on the other β-*h*-fructosidase must split off β-fructose. These two enzymes which occur in association in yeast exhibit their optimal effects at different p_H.

As can be seen from the curves shown in Fig. 21 α-glucosidase is most active nearly at the neutral point whilst β-*h*-fructosidase exhibits optimal activity at p_H 4·7. α-Glucosidase is quite inactive at this point.

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[1] Hitchcock, *J. Amer. Chem. Soc.*, 1926, **48**, 2870; Weidenhagen and Landt, *Z. Ver. deut. Zuckerind.*, 1930, **80**, 25.

SEPARATION OF α-GLUCOSIDASE FROM β-H-FRUCTOSIDASE

Experiment.—Mix 500 g. of pressed bottom yeast with 10 per cent of ethyl acetate, stir for 15 minutes and add 500 c.c. of water. Leave to autolyse for 48 hours at 0°, continually neutralising, with dilute ammonia solution, the acid produced. Then filter or centrifuge at 0°.

Treat 1000 c.c. of the liquid with 325 c.c. of a suspension of fresh aluminium hydroxide B (equivalent to 4·5 g. of Al_2O_3). After 1 hour separate the solid in a centrifuge (the residual solution still contains some α-glucosidase together with much β-fructosidase).

Elute the solid for about 1–3 hours with 580 c.c. of 2 per cent potassium dihydrogen phosphate solution which almost completely removes the β-fructosidase, whilst the bulk of the α-glucosidase remains on the adsorbent. Centrifuge and then separate the α-glucosidase by elution for 30 minutes with 580 c.c. of 2 per cent diammonium hydrogen phosphate solution. Finally, centrifuge once more.

Since the enzymes are very easily destroyed always keep the solutions at 0° and carry out all the operations at low temperature and as rapidly as possible.

In the autolysed liquid, determine the β-fructosidase content of 5 c.c. at p_H 4·7 with sucrose as substrate (proceed exactly as described above for the determination of invertase). At the same time allow 10 c.c. of the liquid to act on maltose at p_H 4·7 according to the method described below. (No hydrolysis or only very little should take place.)

Before making the determination adjust the p_H of the neutral autolysed liquid to 4·7 as follows: Take a small measured volume of the liquid in a test-tube, add a few drops of methyl-red solution and then a measured volume of dilute acetic acid sufficient to produce the colour corresponding to p_H 4·7. Then add to the bulk of the autolysed liquid the calculated amount of acetic acid (naturally without adding any indicator).

In addition, determine at p_H 6·9 the α-glucosidase content of the liquid with (*a*) sucrose (*b*) maltose as substrate.

(*a*) Instead of 5 c.c. of sodium dihydrogen phosphate solution add to the mixture in which the determination is to be made 5 c.c. of 0·33*M*-phosphate buffer solution of p_H 6·9 (2·0 c.c. potassium dihydrogen phosphate and 3 c.c. of disodium hydrogen phosphate).

For the determination use 10 c.c. of liquid. Carry out the determination otherwise as described for the determination of invertase (p. 125).

(*b*) Use the polarimetric method[1] exactly as in the determination of invertase. In order to attain a suitable degree of hydrolysis in the usual reaction time, when determining α-glucosidase in autolysed yeast, use preferably the number of cubic centimetres of solution corresponding to 1 g. of dried yeast. Pour this volume (in this case the best volume is 10 c.c.) together with a solution of 2·5 g. of hydrated maltose and 0·5 g. of phosphate mixture[2] of p_H 6·8 into a 50 c.c. measuring flask containing a few drops of toluene and standing in a thermostat at 30°. Then make up to the mark with water at 30°. Exactly as in the invertase determination, withdraw 25 c.c. after a determined time, note exactly the time when half of this volume has run out of the pipette into the 5 c.c. of $2N$-sodium carbonate solution and polarise in a 2 dm. tube.

The initial rotation in a 2-dm. tube is $+10{\cdot}80°$ and the final rotation, if hydrolysis were complete, would be $+4{\cdot}40°$, the decrease in rotation being 6·40°. If, after 98·3 minutes, for example, the rotation is 7·37° then the decrease in rotation is $10{\cdot}80° - 7{\cdot}37° = 3{\cdot}43°$ and the percentage of hydrolysis is $3{\cdot}43/6{\cdot}40 = 53{\cdot}6$ per cent.

Determine also at p_H 6·9, using sucrose and maltose as substrates, the α-glucosidase content of 10 c.c. of the final solution obtained by elution with diammonium hydrogen phosphate.

At the same time, allow 10 c.c. of this solution to act on sucrose at p_H 4·7. Only a negligible amount of hydrolysis should occur.

DETERMINATION OF AMYLASE ACTION BY THE METHOD OF WILLSTÄTTER AND SCHUDEL

The transformation of higher carbohydrates, such as starch and glycogen, is brought about in the animal and vegetable organism by amylases or diastases. In all cases the final product of the degradation is maltose.

If pancreatic amylase is subjected to extensive dialysis it loses its activity but this is restored by addition of salts, especially chlorides. The diastase present in malt does not require activation by salts.

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[1] Cf. Bamann in Oppenheimer's *Die Fermente und ihre Wirkungen*, vol. iii., p. 855.

[2] Consisting of 0·285 g. of $Na_2HPO_4 . 2H_2O$ and 0·215 g. of KH_2PO_4.

In the animal organism the saliva and the pancreatic juice amongst the digestive secretions—and also the organs which store carbohydrates, for example, the liver—are particularly rich in amylases. In plants also the amylases occur in those parts where starch is most stored and transformed (malt). The lipases and proteases obtained during the preparation of amylases, by extraction of the pancreas with glycerol, for example, can be removed by fractional adsorption on alumina and kaolin.[1]

Determination of Amylase Action.[1]—The reducing groups produced during the hydrolysis of carbohydrate are determined by the hypo-iodite method of Willstätter and Schudel (see p. 76).

The hydrolysis of 0·25 g. of soluble starch by pancreatic amylase in 37 c.c. at 37° is followed until the first 40 per cent hydrolysis has been attained. On the assumption that the reaction is unimolecular the amylase unit is calculated from the following equation :

$$k=\frac{1}{t}\cdot\log_{10}\frac{a}{a-x},$$

in which t is the time, a is the initial concentration and x is the amount of maltose corresponding to the iodine consumed. a is not the actual amount (0·25 g.) weighed out but the amount which, in practice, is saccharified under normal conditions, namely, 75 per cent. Accordingly $a=0{\cdot}1875$ g. In the determination of amylases it is preferable to choose an amount of enzyme such that the constant, k, of the reaction lies between 0·001 and 0·03. The hydrolysis of the 10–30 per cent of the starch then takes place in 7–30 minutes.

The amylase unit is 100 times that amount of enzyme for which, under the given experimental conditions, the constant of the unimolecular reaction is 0·01. Thus the reaction constant expresses the number of amylase units in the sample.

Procedure.—In a measuring cylinder (capacity 50 c.c.) with a ground-glass stopper mix 25 c.c. of freshly prepared 1 per cent soluble starch solution, 10 c.c. of 0·2 N-phosphate buffer solution (consisting of 5·1 c.c. of 0·2 N-potassium dihydrogen phosphate solution and 4·9 c.c. of 0·2 *N*-disodium hydrogen phosphate solution) of p_H 6·8, and 1 c.c. of 0·2*N*-sodium chloride solution (to activate the enzyme) and place in a thermostat at 37°. Add the enzyme (dry material from a weighing bottle or 1 c.c. of solution from a graduated pipette) with shaking. After a short time (usually 10 minutes) arrest the reaction by addition of 2 c.c. of *N*-hydrochloric acid. With a little water wash the reaction mixture into a conical flask and treat with iodine, using 0·6 c.c. of 0·1 *N*-iodine solution for each mg. of maltose expected, and then immediately add, drop by drop (with shaking), 0·1 *N*-sodium hydroxide solution, the volume thus added being such that, after the hydrochloric acid has been neutralised and the acid phosphate of the buffer has been converted into hydrogen phosphate (30 c.c. altogether are required for these two processes), an additional amount 1·5 times the volume of the iodine solution is used. Leave for 15 minutes, acidify with dilute sulphuric acid and titrate the excess of iodine with 0·1 *N*-thiosulphate solution.

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[1] For the preparation of pancreatic amylase see Willstätter, Waldschmidt-Leitz and Hesse, *Z. physiol. Chem.*, 1923, 126, 143. For the preparation of plant amylases see von Euler, and Svanberg, *Z. physiol. Chem.*, 1920, 112, 193.

By means of a blank experiment determine, at the same time, the amount of iodine consumed by the starch and by the solution of the enzyme.

Example.—0·8 c.c. of a solution prepared from 1·0 c.c. of glycerol extract of pancreas by dilution with 250 c.c. of water was allowed to act on starch for 10 minutes under the conditions described above. After the hydrolysis had been stopped by addition of acid, it was found by titration that the aldehydo-sugar produced consumed 2·29 c.c. of 0·1 *N*-iodine solution. A blank determination showed that the starch and enzyme solutions consumed 0·53 c.c. of 0·1 *N*-iodine solution.

The amount of maltose produced was found to be 30·2 mg. calculated from the amount of iodine consumed (1·76 c.c. of 0·1 *N*-solution) and the reaction constant was

$$k=\frac{1}{10}\cdot\log_{10}\frac{0{\cdot}1875}{0{\cdot}1875-0{\cdot}0302}=0{\cdot}0076.$$

Hence the sample used contained 0·0076 units of amylase.

The number of amylase units in 0·1 g. of substance is taken as a measure of the degree of purity of the enzyme preparation.

In the determination of the action of malt diastase, activation with NaCl is not applied and the determination is carried out at p_H 5·6.

3. ESTERASES

The action of esterases consists, in general, of the hydrolysis and synthesis of esters of carboxylic acids according to the equation :

$$R-COO{\cdot}R' + H_2O \rightleftharpoons R-COOH + R'{\cdot}OH.$$

Esters of polyhydric alcohols, for example glycerides of fatty acids, are affected as well as those of monohydric alcohols.

Lipase, which occurs in the pancreas, acts on fats as readily as on lower esters, whilst **liver esterase** from liver very readily attacks lower esters but attacks neutral fats only with difficulty. According to recent investigations by Kraut[1] this difference in activity is to be traced only to differences in the colloidal carrier of the enzyme (the " pheron "), the active group or " agon " being the same in both cases at least within the same animal species.

In addition to the lipases and esterases, a series of specific esterases is known (tannase, phosphatase, chlorophyllase).

PRINCIPLE OF COMPENSATION FOR ACTIVATION AND INHIBITION

The activity of the lipases can be greatly stimulated or inhibited by addition of certain substances. Since such activators or inhibitors often occur in admixture with the enzymes and can be removed to a greater or less extent by further purification, they naturally affect the enzymic reaction in varying but indeterminable degree. Accordingly, before a lipase is determined, compensation must be made for the differences in activity caused by inhibition and activation.

For example, the hydrolysis of oil by pancreatic lipase is greatly affected by impurities which accompany it and by the products of the reaction, whilst the hydrogen-ion concentration is of less importance for the activity. The action of pancreatic lipase on true fats at feebly alkaline reaction is activated by calcium soaps, by salts of bile acids and ovalbumin. But in acid media some of these activators are indifferent and some even inhibitory.

In accordance with the theories of catalysis the activation of lipase is traced to improvement in the adsorptive relations between enzyme and substrate and the inhibition to disturbance of these relations. This is best understood by assuming that contact between the water-soluble enzyme and the insoluble substrate is facilitated by production of colloidal particles which have an adsorbent effect on both enzyme and substrate. At the adsorbent surface, relations between enzyme and substrate can be produced which are more favourable for the reaction than is the direct relation between enzyme and substrate. It has been shown, for example, that calcium soaps adsorb fats on the one hand and enzymes on the other. Such adsorbates of the type

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[1] *Biochem. Z.*, 1935, 276, 114.

Calcium oleate

Lipase Fat

are known as " complex adsorbates ".

The phenomena of inhibition can be explained by supposing that the inhibitor competes with an adsorbent which favours the bringing together of enzyme and substrate.

Any method then which is to be used for the determination of lipase activity (for example, a method to be used during the progressive purification of some enzyme preparation) must, in the first place, exclude from the system all fortuitous factors which would affect the activity. The process of compensation of activation or inhibition makes it possible to measure the activity of lipases under comparable conditions. The hydrolysis can be measured by titration or stalagmometrically.

STALAGMOMETRIC DETERMINATION OF ESTERASES[1]

Lower esters which greatly reduce the surface tension of an aqueous solution but whose products of hydrolysis do so only slightly or not at all are suitable substrates for determination of the saponifying power of enzymes. Thus the change in the surface tension of a solution of tributyrin can be determined stalagmometrically with compensatory activation of the enzyme (addition

[1] Willstätter and Memmen, *Z. physiol. Chem.*, 1923, 129, 1.

of an activating mixture of albumin, sodium oleate and calcium chloride in excess [1]).

Solutions required :

(1) Enzyme solution (blood, serum, extract of organs, filtered gastric juice).

(2) Saturated solution of tributyrin. Shake 50 g. of the commercial, and always impure, tributyrin eleven times (long periods) on a machine with 400 c.c. portions of water. Leave for a short time in a separating funnel until the layers separate, and then run off the water. Now shake twenty drops of the purified tributyrin with 1 litre of water not too vigorously for 1–2 hours in the machine, and leave for 12–24 hours. Never use mixtures more than 2 days old. Allow the undissolved portion to settle and filter through a moist filter paper. Pour the clear filtrate (reject the first portion) into a dropping funnel so that solution may be withdrawn without taking undissolved drops of tributyrin from the surface.

(3) Ovalbumin.

(4) 2 per cent calcium chloride solution.

(5) 2 per cent sodium oleate solution.

(6) Buffer solution : One part of 2·5 *N*-ammonia solution + eight parts of 2·5 *N*-ammonium chloride solution. p_H 8·6 at 18°.

Endeavour to choose the amount of enzyme not appreciably more than twice and not less than one-fifth of a butyrase unit. Take care that after the period of hydrolysis is over, the concentration of tributyrin is still sufficiently great as to fall in the straight portion of the curve of standardisation (see below).

Procedure.—Carry out a preliminary experiment in order to determine the amount of enzyme which is suitable. Mix this amount with a solution of 30 mg. of ovalbumin (usually 0·5–1·0 c.c. of solution), 0·5 c.c. of 2 per cent calcium chloride solution and 56 c.c. of tributyrin solution and add at once 2 c.c. of ammonia-ammonium chloride buffer solution and 0·5 c.c. of 2 per cent solution of sodium oleate so that the total volume amounts to 60 c.c. Then determine, immediately, the initial drop number ; and at a temperature of 20°, at intervals of 20 minutes, repeat the determination three or four times so as to measure the decrease in this number.

For this purpose use Ostwald's straight stalagmometer (see

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[1] Liver esterase, in contrast to pancreatic lipase, is not activated but inhibited by addition of this mixture. Accordingly, with liver esterase, the determination is carried out without activation.

Fig. 22). Essentially this is a pipette which is surrounded by a water jacket in order to maintain constant temperature in the liquid used in the determination.

Determine the number of drops produced by allowing the liquid to sink from one mark to another. This number is directly proportional to the surface tension. First, calibrate the stalagmometer with water at 18°, then with saturated solution of tributyrin; and then for this solution successively diluted with water so that the concentration of tributryin is diminished each time by 10 per cent. Plot on a graph the values so obtained. By means of the curve thus obtained, determine, from the drop number, the concentration of tributyrin still present in the solution after partial hydrolysis by the enzyme. Express the result as a percentage of the concentration of the saturated solution.

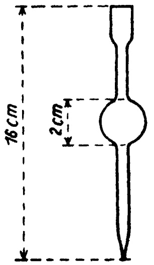

FIG. 22

With the stalagmometer, suck from the flask the amount of liquid to be used, as in filling a pipette, and then determine the drop number in the manner described. Repeat this operation and take the mean of the two values.

The lipase unit for the hydrolysis of tributyrin is defined as that amount which, under the conditions given above, causes a decrease of 20 in the drop number in 50 minutes, *i.e.* a decrease of about half of the difference between the drop numbers of pure tributyrin solution and water.

C. RESPIRATION AND FERMENTATION

I. THE BIOLOGICAL ACTIVATION OF HYDROGEN. THE DEHYDROGENASES

MODEL EXPERIMENTS ILLUSTRATING WIELAND'S THEORY OF DEHYDROGENATION [1]

In the general part (see p. 1 *et seq.*) the reasons which determine the classification of enzymes as hydrolysing and desmolysing were given. Not the least important of these reasons were those concerning questions of energy. Whilst the hydrolysing enzymes serve to initiate the degradation of substances of high molecular weight the desmolysing serve, in the anaerobic stage, to prepare the degradation products for the respiratory process and to effect the respiratory process itself. The characteristic feature of all these desmolytic reactions is the enzymic activation and transfer of hydrogen. Wieland's *theory of dehydrogenation* provides a comprehensive view of this biological catalytic process. The theory is based on model experiments with biological material and inorganic catalysts.

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[1] *Ber.*, 1912, 45, 484. *On the Mechanism of Oxidation* (London, 1932). See also Bredig and Sommer, *Z. physikal. Chem.*, 1910, 70, 34; von Szent-Györgyi, *Studies on Biological Oxidation and Some of its Catalysts* (Leipzig, 1937).

In the first place it is desirable briefly to consider *catalysis* in general and *hydrogenating and dehydrogenating catalysis* in particular.[1] It is characteristic of every catalysis whether in homogeneous or heterogeneous systems that it may be supposed to involve the production of intermediate compounds by means of intermediate reactions, new courses for the reactions being created. In the production of the intermediate compounds the catalysts play an active part. Thus, for example, in catalytic hydrogenation in a heterogeneous system, since a catalyst is always brought into connexion with all of the substances participating in the reaction, the substrate to be hydrogenated *and* the hydrogen are adsorbed on especially *active positions on the catalyst.* These are exactly the positions which are to be regarded as responsible for the catalytic effect: somehow they are excellently adapted for the purpose. The reaction is brought about by changes in the chemical affinities of the adsorbed substances. Accordingly, in catalytic hydrogenation the substrate and the hydrogen are activated. In heterogeneous catalysis with inorganic catalysts it happens only rarely that the *adsorption complex* can be formulated stoichiometrically. But in micro-heterogeneous organic catalyses, such as those due to dissolved enzymes, this is usually possible. We refer here to p. 128, where in the case of the enzymes which attack sugars and proteins the stoichiometrical formulation of the enzyme-substrate complex according to the view of Michaelis and Menten made possible the determination of the affinity constants. The laws which hold for heterogeneous catalysis with inorganic catalysts hold also for the catalytic action of the colloidal particles of the enzymes. With these it is the *active groups in the enzymes* which permit the formation of loose compounds with the substrate because of the specific affinities involved. This holds also for biological catalyses in which bound (and occasionally also free) hydrogen is enzymically activated and transferred, but in these cases active groups must be available in the enzyme for the hydrogenating agent *and* for the substance which is hydrogenated.

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[1] Schwab, *Katalyse vom Standpunkt des chemischen Kinetik* (Berlin, 1931).

The hydrogenation of an organic substance is usually strongly exothermic. As is well known the catalyst causes an equilibrium to be attained which would not be attained, or would be attained only slowly without it. In *hydrogenation*, the reaction leading to the production of the hydrogenation product is very much more favoured by the position of the point of equilibrium than is the reverse reaction. The position of the point of equilibrium varies with change of temperature. If the interacting substances and the products of the reaction are sufficiently stable, the application of heat, by causing the temperature of the reaction to increase, may cause hydrogen to be eliminated from the product of hydrogenation even in the presence of the same catalyst. Accordingly, the hydrogenation process is reversed, and *dehydrogenation* is catalytically accelerated (see the account of the equilibrium *cyclo*hexane-benzene in Gattermann's *Laboratory Methods of Organic Chemistry*, pp. 382–383). On the other hand there also exist substances containing hydrogen which lose it in contact with a catalyst at ordinary temperature.

So far only the addition and elimination of *free* hydrogen have been considered. The energy required for dehydrogenation can also be provided by the *coupling of reactions*. The hydrogen of the substrate AH_2 when activated by the catalyst can be transferred to a compound B which is capable of being hydrogenated although no heat is applied. This happens because the dehydrogenation is coupled with a hydrogenation, that is to say, when the energy produced in the hydrogenation is at least equal to that required for the dehydrogenation, or in other words, when the *free energy* of the whole process, this *oxidation-reduction*, is positive. Accordingly, in the system

$$AH_2 + B \rightleftharpoons BH_2 + A,$$

the reaction proceeds from left to right until equilibrium is attained only when the energy value on the right is positive. In many cases the free energy of a process is approximately equal to its *heat of reaction* which can be determined from the thermal constants of the compounds concerned. But in cases such as that just described an exact measure of the thermodynamic magnitudes involved is obtained by determination of the *oxidation-reduction potentials* (" redox " potentials) of the two systems concerned, namely, A/AH_2 and B/BH_2. (In order to avoid repetition, the detailed account of oxidation-reduction potential is given on page 173 ff.) In the case of hydrogen in very labile combination, as, for example, in the leuco-bases of certain dyes, equilibrium can also be attained *without* the co-operation of any catalyst to activate the hydrogen. In the *dehydrogenation of biological substances*, however, the *co-operation of a catalyst* capable of activating hydrogen is essential in all but a few exceptional cases. Amongst inorganic substances *platinum* and *palladium* are by far the most important catalysts of this kind for dehydrogenation (and hydrogenation). As we shall see, it is possible to demonstrate accurately their power to activate hydrogen. Their rôle as catalysts of dehydrogenation has been clearly shown just as has that of the *dehydrogenases*, the hydrogen-activating and hydrogen-carrying enzymes of living material. Bivalent *iron* and *copper* can likewise activate hydrogen but in most cases their power to do so has not been proved with certainty.

In cases where active hydrogen atoms, or hydrogen atoms activated by a carrier, are liberated in pairs and so converted into molecular hydrogen the production of *hydrogen peroxide* is to be expected. If the hydrogen is added on to substances free from oxygen and capable of being hydrogenated (or reduced) with production of the corresponding hydrogenated compounds, the process, from a chemical point of view, is merely an example of the process generally known as auto-oxidation (auto-oxidative dehydrogenation). The *basis* of Wieland's *theory of dehydrogenation*,[1] a theory which has acquired fundamental importance in biochemistry, is the comprehensive generalisation which includes all processes which involve the transfer of hydrogen.

Wieland's theory of dehydrogenation was based on *model experiments* with biological material and inorganic catalysts (platinum, palladium). It had to be shown that, in such oxidations with oxygen where it was a question of removing combined hydrogen from substrates containing it (*hydrogen donators*), the action of the catalyst consisted in activating the hydrogen and not the oxygen.

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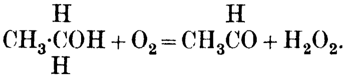
[1] Reviews: Wieland, *Ergebn. Physiol.*, 1922, 20, 477; Bertho, *Ergebn. Enzymforsch.*, 1933, 2, 204; Dakin, *Oxidations and Reductions in the Animal Body* (London, 1922); Thunberg, *Ergebn. Physiol.*, 1937, 39, 76.

The production of hydrogen peroxide, which in itself provides proof, can only be occasionally observed because inorganic catalysts have a powerful (catalase-like) effect on the decomposition of this substance (see p. 146). Wieland provided a more fundamental proof by employing, in a series of cases, other *hydrogen acceptors* in place of oxygen, such as quinone and methylene-blue,[1] and with them achieved the same purpose, namely, oxidation, as with oxygen itself. Such catalytic dehydrogenation whether by oxygen or by " foreign " hydrogen acceptors (*i.e.* acceptors not normally occurring in the cells, such as the quinonoid compounds mentioned) in the presence of inorganic catalysts is the artificial counterpart of natural biological oxidation-reduction in which the dehydrogenases play their part and hence the *model reactions* are of especial significance.

Let us consider some of these examples :

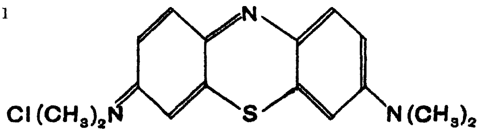
As Schönbein observed long ago *alcohol* in the presence of spongy *platinum* is oxidised by atmospheric oxygen. From the fact that in this process quinone and methylene-blue can act as acceptors being thereby converted into quinol and leucomethylene-blue respectively it follows that the reaction is a dehydrogenation which proceeds according to the equation :



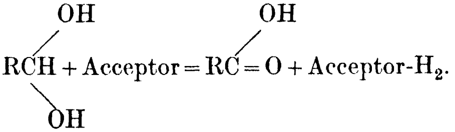
$$CH_3{\cdot}\overset{H}{\underset{H}{C}}OH + O_2 = CH_3\overset{H}{C}O + H_2O_2.$$

The catalase-like action of the platinum prevents the detection of the hydrogen peroxide in this case. In a similar way *lactic acid* may be catalytically oxidised by *spongy palladium* to acetic acid and carbon dioxide and *glucose* to carbon dioxide and water ("oxidation without oxygen"). Similarly also, the conversion of *aldehydes* into acids in the presence of palladium black is a dehydrogenation although, at the first glance, this does not seem comprehensible. If account is taken of the fact that this reaction takes place only when water is present it is evident that the oxidation of

[1]



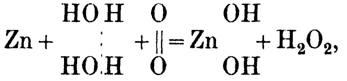
aldehydes is a dehydrogenation of the hydrated aldehyde according to the equation :



$$\mathrm{RCH}\begin{matrix}\diagup \mathrm{OH}\\ \diagdown \mathrm{OH}\end{matrix} + \text{Acceptor} = \mathrm{RC}\begin{matrix}\diagup \mathrm{OH}\\ \end{matrix}\!\!=\mathrm{O} + \text{Acceptor-}H_2.$$

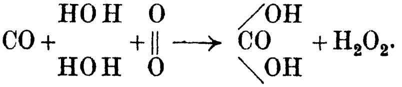
For this reason also *chloral*, in contrast to its hydrate, does not react with oxidising agents in anhydrous solvents. The catalytic oxidation of *carbon monoxide* by platinum also requires the presence of small amounts of water. In the absence of a catalyst this oxidation occurs only at high temperature. According to Wieland and Wartenberg, formic acid (hydrate !) is an intermediate product in the combustion of carbon monoxide. The acid is then dehydrogenated to carbon dioxide whilst the oxygen consumed reappears, by way of hydrogen peroxide, as water which can again take part in the reaction and hence acts as a catalyst.

At this point it may be mentioned that Traube regarded the combustion of carbon monoxide as the most important organic example for the support of his theory of vital oxidation. It is well known that base metals undergo auto-oxidation only in presence of water, *i.e.* they cannot decompose water without the co-operation of oxygen. According to the equation :



$$\mathrm{Zn} + \begin{matrix}\mathrm{HO\,H}\\ \vdots\\ \mathrm{HO\,H}\end{matrix} + \begin{matrix}\mathrm{O}\\ \|\\ \mathrm{O}\end{matrix} = \mathrm{Zn}\begin{matrix}\mathrm{OH}\\ \\ \mathrm{OH}\end{matrix} + H_2O_2,$$

the affinity of hydrogen for oxygen causes this hydrogenation leading to the production of zinc hydroxide (coupled reactions). Here the oxygen can be replaced by other acceptors provided that their affinity for hydrogen is sufficiently great. The oxidation of carbon monoxide which does not occur in dry air has likewise been attributed (by Traube) to such a decomposition of water :



$$\mathrm{CO} + \begin{matrix}\mathrm{HO\,H}\\ \\ \mathrm{HO\,H}\end{matrix} + \begin{matrix}\mathrm{O}\\ \|\\ \mathrm{O}\end{matrix} \longrightarrow \mathrm{CO}\begin{matrix}\diagup \mathrm{OH}\\ \\ \diagdown \mathrm{OH}\end{matrix} + H_2O_2.$$

This view has been rendered untenable by the detection of formic acid as an intermediate product in the combustion of carbon monoxide (see above). Here also, rather, we have a dehydrogenation.

Such model reactions of biological dehydrogenation extend to *amino-acids* also. With palladium or *activated carbon* as catalysts they can be dehydrogenated. Alloxan, *m*-dinitrobenzene, quinone and dithiodiglycollic acid may be used as hydrogen acceptors in this case. It is probable that the dehydrogenation takes place by way of the imino-acid which, by decarboxylation, is converted into the imino-aldehyde which finally decomposes into ammonia and aldehyde. The production of keto-acid is not a stage in the process because palladium cannot decarboxylate keto-acids.

Cases are also known where hydrogenated compounds, such as hydrazobenzene, hydrogenated naphthalenes and so on, for example, are dehydrogenated by contact with platinum or palladium in the absence of any hydrogen acceptor, the hydrogen being taken up by the metal. From what has been said above, such behaviour of hydrogenated products is only possible, on thermodynamic grounds, when the dehydrogenation occurs with loss of energy and this is usually not the case.

The behaviour of **iron** and **copper** is much more difficult to explain than is that of the noble metals. We know that oxidations, in particular, can be catalysed by iron, the process being more or less similar to biological oxidation. In a few cases only, however, can it be definitely decided that activation of hydrogen occurs. Such are the cases where oxygen-free acceptors are involved. In by far the greatest number of cases of catalysis by iron it is entirely a matter of opinion whether one supposes that the heavy metal or a heavy metal-substrate complex has the power to activate the hydrogen of the substrate (Wieland) or that the oxygen is prepared to carry out the oxidation (Warburg).[1] Here, already in connexion with these model reactions, we encounter the question concerning cellular oxidation which has not yet been definitely answered. Possibly the question is pointless, because, as was mentioned at the start, it is essential for the initiation of every catalytic reaction that both participants—the substrate which is to be dehydrogenated *and* oxygen or another acceptor—must come into special relation with the catalyst. Observation of enzymic dehydrogenation favours such a view (see p. 196). These considerations naturally do not apply to catalytic auto-oxidations which occur without any transfer of hydrogen. In these cases it is a question of the uptake of oxygen at the double bonds of the substrate (catalytic auto-oxidation of linolenic acid and lecithin, see p. 221).

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[1] These model experiments also fulfil the requirement of Wieland's theory, namely, that the action of the oxidases (enzymes which activate oxygen) actually is that of hydrogen-activating dehydrogenases.

Only the most striking examples can be considered in detail here. An indisputable case of dehydrogenation catalytically accelerated by an iron salt is the *dehydrogenation by methylene-blue,* in the presence of a *ferrous salt, of cysteine and thioglycollic acid* with production of cystine and dithiodiglycollic acid on the one hand and leucomethylene-blue on the other (MB = methylene-blue):

$$2RSH + MB = RSSR + MBH_2.$$

These reactions were studied by Toda and Harrison.

In cases where *oxygen* is the oxidising agent the production of *hydrogen peroxide* must serve as proof of the fact of dehydrogenation. Thus the spontaneous auto-oxidation of *quinol, catechol, leucomethylene-blue* and so on *in the absence of metals* are shown to be dehydrogenations by the production of hydrogen peroxide. When bivalent or trivalent iron is added these auto-oxidations become *catalytic auto-oxidations.*[1] However, the detection of hydrogen peroxide in processes catalysed by iron is not possible for two reasons, quite apart from the fact that mechanism of the reaction undergoes alteration in some way. In the first place *iron salts*, just like the *platinum metals* mentioned above, have a powerful decomposing action (*catalase action*), and in the second they cause a secondary reaction, namely, the interaction of the hydrogen peroxide and the organic substrate (*peroxidase action,* see below). The catalase and peroxidase actions of copper are not so powerful. The catalytic oxidation of leucomethylene-blue in the presence of copper salt involves the production of hydrogen peroxide and accordingly the process is definitely a catalytic dehydrogenation (*dehydrogenase model*).

In the catalytic auto-oxidations mentioned, it suffices to suppose that the iron produces its catalytic effect by means of continual *conversion of the ferrous into the ferric form* and *vice versa,* the substrate being oxidised by the ferric form and the ferrous form undergoing continuous auto-oxidation. But this supposition is inadequate to explain a number of other cases of catalytic auto-oxidation. According to Wieland, together with the mechanism described, there is a production of *iron-substrate complexes* in which the hydrogen of the substrate is activated and so adapted for the uptake of molecular oxygen. The hydrogen peroxide here produced in the first instance is then consumed in the peroxidase reaction (see below).

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[1] Wieland and Franke, *Annalen,* **1928, 464, 101; 1929, 475, 19.**

The auto-oxidation of the ferrous iron and the accompanying inactivation of the catalytic complex is supposed to be prevented because the ferrous-ferric catalytic process mentioned above continues at the same time. Warburg,[1] on the other hand, bases his theory of oxygen activation (see p. 222) exclusively on the action of such metal-substrate complexes but ascribes to them the power to activate oxygen somewhat as in the *catalytic auto-oxidation of cysteine* and of many *carbohydrates*. Thus, in these cases, a clear distinction between " dehydrogenase " and " oxidase " is not possible. The distinction could be made if success were attained in the attempt to detect hydrogen peroxide but, on account of the catalase and peroxidase actions of iron, this cannot be done.

The peroxidase action of the ferro-substrate complex can also be studied alone if hydrogen peroxide is used instead of oxygen as oxidising agent.[2] The *oxidation of the dihydric phenols, of* p-*phenylenediamine and of the thiol-compounds by hydrogen peroxide* in the presence of ferrous salt or copper is a true catalysis (peroxidase model). Here, according to Wieland, the hydrogen acceptor, hydrogen peroxide, takes up the activated hydrogen of the ferro-substrate complex with production of water. From this alone it is evident that the above-mentioned catalysis by oxygen, which is caused by metal-substrate complexes is, in its second stage, nothing else than catalysis by hydrogen peroxide. Such metal-substrate complexes, indeed, can even be definitely detected. Küchlin and Boeseken have investigated the *catalytic oxidation of carbohydrates by hydrogen peroxide* in the presence of ferrous salts and have definitely detected the existence of an active complex of the kind described in the case of the oxidation of fructose. Further contributions to the study of this subject have been made by Franke. Particularly interesting is the fact that, according to the former authors, complex catalysts of this kind are not non-specific oxidising agents but exhibit specificity just as does the enzyme peroxidase (see p. 208).

The significance of the haemins as catalysts of oxidation is discussed later (see pp. 207, 222).

It is frequently desired to decide whether an auto-oxidation proceeds spontaneously in the absence of metals or whether the uptake of oxygen is caused by traces of heavy metal having catalytic activity. Such traces may be present as impurity in the substrate and often can be removed only by means of extraordinarily careful purification (sublimation, and so forth). The *sensitivity* of the reaction to the action of *hydrocyanic acid* is then tested. Catalyses by iron are usually sensitive to the action of this acid, whilst catalyses by copper are considerably less sensitive. By poisoning the catalyst (production of a complex) the consumption of oxygen is reduced and so an indication is given of the possible presence of metal. Cases are known where this criterion fails. Inhibition by *carbon monoxide* can often be successfully applied.

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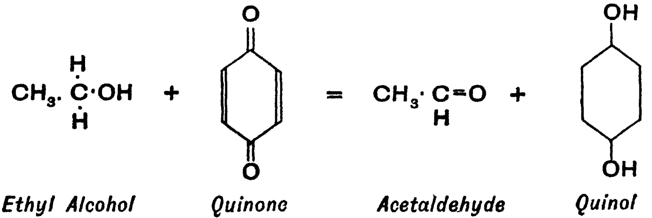
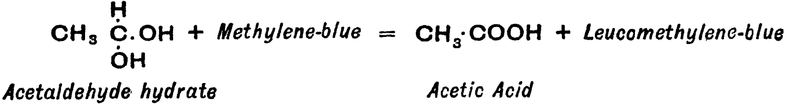
[1] *Über die katalytische Wirkung der lebenden Substanz* (Berlin, 1928).

[2] Wieland and Franke, *Annalen*, 1927, 457, 1 ; 1929, 475, 1.

BIOLOGICAL DEHYDROGENATION. METHOD OF USING ACCEPTORS

The fact that quinone and methylene-blue are able, in presence of acetic bacteria, to convert ethyl alcohol and acetaldehyde into acetic acid in the absence of oxygen, and hence to act as substitutes for oxygen,[1] provides the starting point for the application of Wieland's dehydrogenation theory to biochemistry. In the same way





$$CH_3\overset{H}{\underset{OH}{C}}.OH + \text{Methylene-blue} = CH_3\cdot COOH + \text{Leucomethylene-blue}$$

as in the model reactions, the action of platinum and palladium was explained as being that of catalysts which activate hydrogen, so specific enzymes in the cells, namely, the *dehydrogenases* (*hydrokinases*), are believed to mobilise pairs of combined hydrogen atoms in the substrate (hydrogen donators), that is to say, to activate these atoms, which then become available for combination with oxygen, quinone, methylene-blue and indeed any hydrogen acceptor at all in the same manner in each case. Since this discovery was made a great number of investigations has been carried out bringing a wide range of evidence in support of the theory. Today, numerous dehydrogenases are known partly only by the action which they exert in co-operation with tissues ; but partly also in numerous cases in the form of solutions of enzymes (succino-dehydrogenase, xanthine-oxidase and so on) for the dehydrogenases are very sensitive enzymes and cannot be brought into solution free from cells with the same ease with which many lipolytic and proteolytic enzymes can be dissolved.

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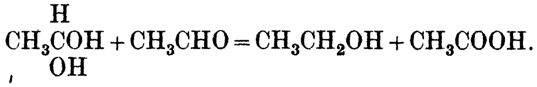
[1] *Ber.*, 1913, 46, 3335.

When it had been found possible, in oxidations occurring in cells (aerobic processes—respiration, oxidative fermentations), to replace the oxygen by foreign acceptors (quinone, methylene-blue and so forth), Wieland's theory of the dehydrogenation process soon proved to be exceptionally fruitful, for it was found to be applicable also to those processes in the metabolism of cells, which proceed in the absence of air (anaerobic processes—mutation reactions, fermentation, glycolysis). Here the conception of the biological transfer of hydrogen, which is characteristic of the nature of these processes, provided a general basis for their understanding. As the simplest example of this kind, the dismutation of acetaldehyde may be mentioned. The enzyme concerned in this process, namely, *aldehyde mutase*, must, according to Wieland's original theory, be identical with *aldehydrase* (see p. 170) since the question of the acceptor does not arise.



$$CH_3\overset{H}{\underset{OH}{C}}OH + CH_3CHO = CH_3CH_2OH + CH_3COOH.$$

Acetaldehyde Hydrate Acetaldehyde Ethyl Alcohol Acetic Acid

In contrast to Warburg's view, which can apply only to the purely *aerobic* process, *respiration*, because it lays chief stress on the activation of oxygen in systems containing iron (haemin-like respiratory enzyme, see p. 222), the dehydrogenation theory applies also to the *anaerobic* processes of the cell which take place during the so-called mutation reactions involving the transfer of hydrogen. Thus the dehydrogenation theory is more comprehensive and covers the processes of fermentation and glycolysis. Whether the process of dehydrogenation of the respiratory substrate is to be regarded as the whole of the *respiratory process* or whether, from case to case, other additional mechanisms are involved, no matter. The course of the dehydrogenation is not in any way made questionable thereby because in various cases the active dehydrogenases can be prepared in a more or less pure condition. Dehydrogenation by oxygen, the chief acceptor in the processes which occur in the cells, proceeds as follows :

$$\underset{\text{Respiratory substrate}}{XH_2} + O_2 \longrightarrow X + H_2O_2 \quad (H_2O_2 \xrightarrow{\text{Catalase}} H_2O + \tfrac{1}{2}O_2).$$

What holds for the model reactions described on p. 143 *et seq.* holds here also, the process catalysed by a dehydrogenase proceeding spontaneously. The gain in energy in the hydrogenation exceeds the expenditure of energy in the dehydrogenation. The energy gained in respiration with oxygen serves, of course, to maintain the life processes of the organism in which the respiration occurs.

According to the dehydrogenation theory **hydrogen peroxide** is produced, in oxidations in the cell, as the primary result of the hydrogenation of *molecular* oxygen, *i.e.* in cases where oxygen is the acceptor. This hydrogen peroxide, it is believed, is decomposed into water and oxygen and so, as is necessary because of its toxicity, removed from the cells by the *catalase* (see p. 201) which is widely distributed in organisms which consume oxygen. And thus, for the first time, it was possible to ascribe to the catalases a general functional significance in the metabolism of the cell.

It was natural to think of replacing oxygen, the most important biological acceptor of hydrogen, by *foreign* substances which are capable of taking up hydrogen because, in the processes which occur in cells, a series of such acceptors is normally active. Such are : the hydrogen acceptors which take part in anaerobic dismutation reactions, namely, compounds containing carbonyl or aldehyde groups or substances containing double bonds—further nitrates, hydrogen peroxide and so on—and certainly also a series of substances which still remain unknown. The dehydrogenated thiols, such as cystine and, especially S·S-glutathione, apparently do not serve as acceptors (Dixon, Bertho). In the cases of these substances either the oxidation-reduction potentials are unfavourable (see p. 173) or the dehydrogenases, because of their specificity towards acceptors, cannot use these compounds as such (see p. 196).

In the study of hydrogenation and dehydrogenation in biological media, that is to say, by dehydrogenases or in dehydrogenation processes modelled on the biological type but employing foreign acceptors, knowledge of the *affinities* is a valuable help.

As was pointed out above (see p. 141) this knowledge can be obtained, in good approximation, by study of the *heats of reaction* derived from thermochemical data, or more exactly by study of *oxidation-reduction potentials.* A special section (see p. 173) is devoted to these potentials. Here the significance of the heat of reaction will be only briefly considered. In the process

$$AH_2 + B \longrightarrow BH_2 + A + x \text{ Cal.}$$

heat is liberated. The thermochemical data necessary for calculating how much are known in most cases (heats of formation and combustion). Some examples will illustrate the point :

$$\text{I. } \underset{\substack{\text{Heat of dehydrogenation of alcohol}}}{\underset{21 \text{ cal.}}{\text{Alcohol} + \text{Quinone}}} \longrightarrow \underset{\substack{\text{Heat of hydrogenation of quinone}}}{\underset{42 \text{ cal.}}{\text{Acetaldehyde} + \text{Quinol}}}$$

The difference is 21 calories, and hence—provided that an alcohol-dehydrogenase is present—the reaction from left to right is possible.

$$\text{II. } \underset{\substack{\text{Heat of dehydrogenation of Succinic}\\ \text{Acid}}}{\underset{31 \text{ cal.}}{\text{Succinic Acid} + \text{Acetaldehyde}}} \longleftarrow \underset{\substack{\text{Heat of hydrogenation of}\\ \text{Alcohol}}}{\underset{21 \text{ cal.}}{\text{Fumaric Acid} + \text{Alcohol}}}$$

According to the thermochemical data only the reaction from right to left is possible since, otherwise, an expenditure of energy equivalent to 10 calories would be required.

In the following table such thermochemical data are arranged systematically. Column 4 gives the differences in the heats of combustion or, which is the same thing, the heat of dehydrogenation by O_2. Column 2 shows the heat of hydrogenation or dehydrogenation which is the difference between the heat of combustion by O_2 of the free H_2 molecule (68·4 calories) and the heat of dehydrogenation by O_2. The heat of hydrogenation is naturally equal to the difference between the heats of formation. The thermochemical data agree only approximately with the maximal amount of work done. This is evident since the results which should follow from the above calculations frequently do not correspond with the actual facts. For example, succinic acid hydrogenates methylene-blue in the presence of succino-dehydrogenase although the calculated heat of reaction is $-5{\cdot}6$ calories. Accordingly, the methylene-blue should not be hydrogenated. The heat of dehydrogenation of succinic acid differs in this case very considerably from the free energy, whilst the affinity involved in the hydrogenation of methylene-blue, which can be calculated exactly from the oxidation-reduction potential, corresponds very well with the heat of reaction.

Hydrogen Acceptor	Heat of Hydrogenation. Cal.	Hydrogenation Product	Heat of Dehydrogenation by O_2 Cal.
Potassium persulphate	+100	Potassium hydrogen sulphate	−32 (2 mol.)
Hydrogen peroxide	+92	Water	−24 (2 mol.)
Oxygen	+45	Hydrogen peroxide	+23
Quinone	+42	Quinol	+26
Oleic acid	+38	Stearic acid	+30
Ethylene	+37	Ethane	+31
Fumaric acid	+31	Succinic acid	+37
Salicylaldehyde	+30	Saligenin	+38
Acetaldehyde	+21	Ethyl alcohol	+47
Nitrogen	+22	Ammonia (2 mol.)	+91.5 (per mol.)
Succinic acid	+ 6	Acetic acid	+62 (2 mol.)

In the **acceptor technique** so-called foreign hydrogen acceptors (briefly, acceptors) are used. These have properties (*e.g.* capability to be determined by chemical methods, solubility, colour and so on) which enable them to play an intimate part in the dehydrogenation process. They are used with a view to reproduce, as far as possible, the processes in the cell which occur in the absence of oxygen but involve transfer of hydrogen (dismutation reactions), or else in order to compare actual respiration (with oxygen) with the respiration which occurs when acceptors are used instead. The most important substances which serve as foreign acceptors are quinonoid compounds which, when hydrogenated, are converted into benzenoid compounds. These quinonoid compounds include dyes of the thiazine series of which methylene-blue is used especially often. Indophenols, quinones and other compounds are also used. In the course of the descriptions of the experiments dealing with methylene-blue technique and oxidation-reduction potential the circumstances which cause the action of foreign acceptors never to simulate completely the nature of the biological process will be discussed.

1. THUNBERG'S METHYLENE-BLUE TECHNIQUE.[1] THE SUCCINO-DEHYDROGENASE OF MUSCLE

In the endeavour to determine the reducing power of tissues, colorimetric acceptor methods have been worked out which depend on the fact that a given amount of an acceptor is consumed by a particular substrate, the progress of the reaction being followed, in serial experiments, by means of a change or loss of colour. Thunberg introduced the use of methylene-blue as acceptor. In his method the times required for the decolorisation of various samples are determined—that is to say, the times which elapse until, with a definite amount of enzyme, definite concentration of donator and definite additions, a given amount of methylene-blue is converted in a vacuum, into leucomethylene-blue. Then when equal amounts of methylene-blue are used, the intensity of reduction is inversely proportional to the decolorisation time. Intermediate stages in the decolorisation are not determined (say by colorimetric comparison measurements). Not the least important reason for this is that the intensity of the colour of methylene-blue is so great that such measurements would not be possible with the amount of the dye used. The methylene-blue method is easily brought into relation with Wieland's theory, for the results of the method correspond to expressions of the reducing power of the cell and, formally, the method takes account of the fundamental elements of Wieland's theory, namely: dehydrogenase, action of the donator, action of the acceptor.

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[1] Ahlgren, *Dissertation* (Lund, 1925).

The methylene-blue method is suitable for testing whether certain donators are attacked by one and the same dehydrogenase. Further, it enables a study to be made of the effect of certain additions on the respiration of cells, particularly when amounts of oxygen cannot be determined manometrically (see p. 186) on account of their small volumes, for the intensity of colour of methylene-blue is extraordinarily great even in low concentrations. The method yields values, in other words, times required for decolorisation which are proportional to the extent of the reaction and may be directly related to the corresponding natural processes in the cells. These values, accordingly, give information about the correlative intensities of oxidation. But they give no absolute measure of the respiratory capacity of a system and consequently also no stoichiometrical values corresponding to the oxygen consumption. In general, the results obtained by the methylene-blue method are consideraby lower than those expressing the oxygen consumption.

Preparation of Muscle Pulp.[1]—The *succino-dehydrogenase* of muscle can be isolated only with difficulty. It rapidly loses its activity in solution. Accordingly, it is preferable to use the enzyme-containing material for experimental purposes.

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[1] Fischer, *Ber.*, 1927, 60 [B], 2260; Wieland and Frage, *Annalen*, 1930, 477, 1.

From a freshly slaughtered horse take the muscle from the left ventricle of the heart, remove the fat and tendons, cut into strips and pass three times through a mincer. Place the minced material in a large filter jar, stir for some minutes with twenty parts by weight of distilled water saturated with toluene, allow to settle and separate from the wash-liquor by decantation. Then repeat the washing twice. Filter as dry as possible with suction. In this way there is obtained in a short time a rather stable preparation containing 16–17 per cent of dry material and exhibiting only a very small "residual respiration" but capable, however, of taking up very considerable amounts of oxygen in the presence of succinate. Keep the material sterile by adding a few drops of toluene and store in the refrigerator.

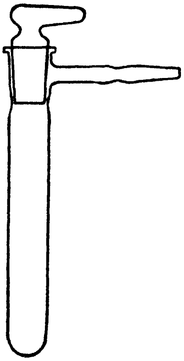


FIG. 23

The Thunberg Apparatus.—At least ten Thunberg tubes (see Fig. 23) are required: they can be purchased ready-made. These are small tubes about 10 cm. long, shaped like filter tubes but having well-fitting ground-in stoppers. The hollow barrel of the stopper has a small hole so placed that when the stopper is turned, until the hole covers the opening of the side tube, connexion is made with the external atmosphere. When the stopper is in any other position, the external atmosphere is completely cut off. The stoppers are carefully greased with "high vacuum" fat or with a mixture of vaseline and beeswax. Also required are a water thermostat fitted with a thermo-regulator and stirring apparatus, and small metal-clips in which the tubes can be fixed so that they project only a little way above the surface of the water.

Experiment.—Study the intensity of reduction in the system succinic acid - succino - dehydrogenase - methylene - blue. Fill the various tubes as shown in the table. Tubes 1 and 2 serve for determining the residual respiration.

Procedure.—It is necessary to use each sample in duplicate or triplicate in order to allow for possible mishaps due to lack of gas-tightness, etc., in one or more of the tubes. Clean the tubes well, grease the stoppers, place in a test-tube stand and pour in the muscle pulp (weighed on a hand-balance), buffer solution, succinic acid solution, methylene-blue solution (Merck's medicinal methylene-blue with $5H_2O$, molecular weight 409·5) and sufficient water to make the volume the same in all the tubes. Add the methylene-blue solution *last* from a pipette shortly before the tubes are ready so that it forms the uppermost layer in each case.

Samples :

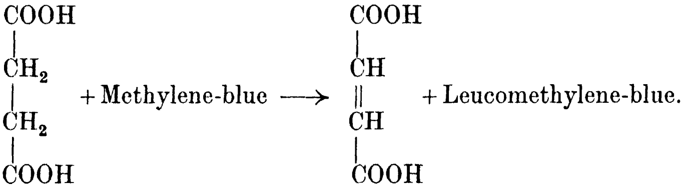
Tube No.	1 2	3 4	5 6	7 8	9 10
Muscle pulp in g.	0·1	0·1	0·1	0·1	0·2
0·2N-Phosphate buffer solution, p_H 6·8, in c.c.	1	1	1	1	1
0·01N-Succinic-acid solution, in c.c. . .	—	1	1	2	1
Water, in c.c.	2	1	0·8	—	1
Methylene-blue solution (0·5 mg. per c.c.), in c.c.	0·2	0·2	0·4	0·2	0·2

A short time after adding the methylene-blue, evacuate the tubes successively with a water-jet pump for 1 minute at the temperature of the hand. This causes the contents to boil. Turn the stoppers, so closing the tubes, shake and place in the thermostat at 37°. The evacuation removes atmospheric oxygen which would otherwise re-oxidise the leucomethylene-blue which is produced and the heat of the hand causes production of water-vapour which removes the last traces of oxygen. When it is remembered that 2·7 c.c. of oxygen are required for the oxidation of 0·1 mg. of leucomethylene-blue (H_2O_2 is first produced but is immediately destroyed by the catalase present in the muscle pulp) it is evident that the most careful work is an essential condition for success. Indeed, care and extreme cleanliness are fundamental conditions in all satisfactory biochemical work.

Note, for each tube, the time when the stopper is turned to close the tube or the time when the tube is shaken. This is the *start of the decolorising period.* From time to time shake the tubes while in the thermostat, especially if sediment is produced. When the contents have acquired a just noticeable faint blue shade note the time which is the *end of the decolorising period.* It is not desirable to wait until a shade completely matching that of the initial mixture before addition of the methylene-blue has been attained. A moderately experienced observer can make parallel determinations which, when the decolorisation period is short (about 30 minutes), differ by, at most, 2 minutes and when it is long (about 100 minutes) by 5 minutes at most.

If air is allowed to pass into the tubes the leucomethylene-blue produced undergoes auto-oxidation by the atmospheric oxygen. The greater the alkalinity of the medium, the greater is the rate of this auto-oxidation (H_2O_2, see above).

The dehydrogenation of *succinic acid* by methylene-blue or oxygen in co-operation with the *succino-dehydrogenase* of muscle with production of *fumaric acid* (Dakin, Thunberg) proceeds thus :



$$\begin{array}{l}\text{COOH}\\|\\\text{CH}_2\\|\\\text{CH}_2\\|\\\text{COOH}\end{array} + \text{Methylene-blue} \longrightarrow \begin{array}{l}\text{COOH}\\|\\\text{CH}\\\|\\\text{CH}\\|\\\text{COOH}\end{array} + \text{Leucomethylene-blue.}$$

When muscle pulp is used, up to 75 per cent of the succinic acid consumed can be recovered as fumaric acid. The rest appears as malic acid because of the presence of a *hydratase* which converts fumaric acid into malic acid (Battelli and Stern, F. G. Fischer). The succino-dehydrogenase, which can dehydrogenate no other compound except succinic acid and methylsuccinic acid (Thunberg) can be eluted from the muscle pulp by making a press-juice or by treating with alkaline phosphate buffer solution ($0{\cdot}066M$-Na_2HPO_4, p_H 9, Ohlsson). Such a solution of the enzyme gives, with succinic or fumaric acid, a *reversible oxidation-reduction system* (see p. 182) of which the potential can be measured with a potentiometer. In presence of methylene-blue complete decolorisation is not achieved because a dynamic equilibrium, succinic acid + methylene-blue $\rightleftharpoons$ fumaric acid + leucomethylene-blue is set up. Accordingly, succino-dehydrogenase is a genuine catalyst which acts in the same way *in vivo* and *in vitro*, its action being independent of its origin (horse muscle, ox heart, *B. coli*). In presence of toluene the power to cause dehydrogenation with oxygen is lost. Whether or not the malic acid of muscle undergoes further transformation, depends on the age of the material. The experiment described above should have the following result :

Tubes 1 and 2, if decolorised at all, should show by far the longest periods of decolorisation. In them the "*residual reduction*" is determined. Since even well-washed tissue, which has thus been, to a great extent, freed from crystalloid donators, contains hydrogen-yielding substances—for the most part SH-compounds—it exhibits a more or less pronounced reducing power. Boiled tissue exhibits the same property although to a smaller extent. But in this case the reduction is purely chemical and occurs without the co-operation of an enzyme. The so-called residual reduction is prevented by extracting the washed and boiled tissue with alcohol. Doubtless the phenomenon is a complicated one. Tubes 5 and 6 show a decolorisation period not quite twice as great as that shown by tubes 3 and 4, although the former contain twice as much methylene-blue as do the latter. This is due to the fact that the decrease in the concentration of the dye per unit of time is not uniform. If the values (concentrations of dye as ordinates, times as abscissae) were plotted, the curve obtained would become asymptotic as the concentration of dye decreased. When the concentration of the dye is low this effect is relatively more prolonged than when it is high. The dye and its leuco-product are adsorbed on the material of the tissue and diffusion of the dye and the leuco-product in the cells towards and away from the places where the enzyme is localised occurs ; hence it is very difficult to evaluate such curves kinetically. If a micro-heterogeneous system could be used, *i.e.* if the experiment were carried out with solutions of the enzyme, these difficulties could be avoided. Tubes 7 and 8 which contain twice as much donator as do tubes 3 and 4 show, in general, decolorisation times which are only a little or not at all shorter than those of tubes 3 and 4, because the amount of donator is already optimal in the latter. When the amount of enzyme is doubled (tubes 9 and 10) the decolorisation period is reduced to about half. This conforms to the *enzyme-time law* according to which, when the concentration of substrate is optimal, the product of the amount of enzyme and the period of reaction is constant.

In order to decide whether or not, in such cases, the process is one of diffusion the temperature coefficient, k_{100} of the decolorisation reaction is determined. It lies below 1·3 for diffusion reactions. Normally the temperature coefficient for decolorisation of methylene-blue is 1·8 to 2·0.

In the method described it is not possible to make subsequent additions to the substrate. Further, certain amounts of easily volatile material (*e.g.* aldehyde, hydrocyanic acid and so on) cannot be retained because of the evacuation. Finally, it is often desirable to keep the non-homogeneous contents of the tubes in motion and this also cannot be done in this method. The method described below does not suffer from these defects.

THE DEHYDROGENASES OF MUSCLE TISSUE

The best known and, on account of its pronounced specificity, the most easily accessible of these dehydrogenases is the *succinodehydrogenase* just considered.

The cardiac muscle of the freshly killed animal is able, with oxygen as well as with methylene-blue, to convert the *malic acid* (produced from the fumaric acid) into *pyruvic acid* by way of *oxalacetic acid* (I—V) (Hahn[1]). The dehydrogenases concerned in these changes have not yet been individually studied. In the transformation of pyruvic acid two routes are possible: (1) Decarboxylation to *acetaldehyde* and dehydrogenation of the *acetic acid* produced from it with production of *succinic acid*. It has not yet been possible to detect such production of succinic acid in muscle. In yeast it has been possible (Wieland and Sonderhoff, see p. 247) so that there is a probability that some such process also occurs in muscle. (2) Dehydrogenation of the pyruvic acid to *αα′-diketoadipic acid* which then decomposes into succinic acid and *formic acid* which finally break down into *carbon dioxide* and *water* in the manner shown in the scheme. This view is supported by the discovery that in the surviving liver of the dog, pyruvic acid is converted into succinic acid (Toenissen and Brinkmann). Free αα′-diketoadipic acid has not yet been isolated or synthesised. Since it has not been possible to prepare this relatively simple substance, it is probably very unstable. If so, this accords with its function as a labile intermediate product.

Lactic acid also is dehydrogenated by a specific lactic acid dehydrogenase of muscle (Hahn, Szent-Györgyi). In this process pyruvic acid but no acetic acid is encountered although the latter occurs in the cell (see above). This supports the second of the possibilities mentioned above. The individual enzymes which take part in the transformations beginning with malic acid are, as was already mentioned, not known in detail. Holmberg, however, has shown that the material obtained by extracting horse muscle with phosphate solution is able, together with the co-enzyme of lactic acid dehydrogenase (p. 183) to dehydrogenate malic acid.

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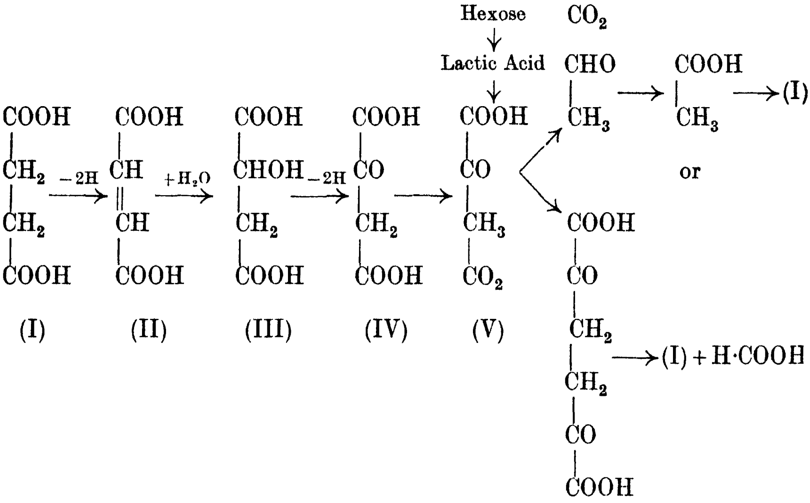
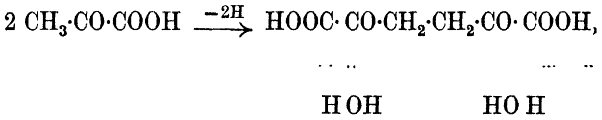
[1] *Z. Biol.*, 1932, 93, 121.





$$2\,CH_3{\cdot}CO{\cdot}COOH \xrightarrow{-2H} HOOC{\cdot}\underset{\text{H OH}}{CO}{\cdot}CH_2{\cdot}CH_2{\cdot}\underset{\text{HO H}}{CO}{\cdot}COOH,$$

Lactic acid and pyruvic acid form connecting links with the *anaerobic part of carbohydrate metabolism.*

Quinol also acts as a hydrogen donator towards muscle. Separation of the succino-dehydrogenase from the quinol dehydrogenase of the press-juice of muscle has already been achieved.

A dehydrogenase, which, besides dehydrogenating acetaldehyde in co-operation with oxygen or methylene-blue, is able to bring about the Cannizzaro reaction with acetaldehyde, is the so-called *liver enzyme.* This dehydrogenase of liver can be brought into solution. Further, there is also present in liver and muscle an alcohol dehydrogenase.

From muscle and also from liver which has been treated with acetone there can be separated a dehydrogenase which, in the presence of methylene-blue, but not of oxygen, dehydrogenates *hexose-diphosphoric acid.* Liver contains also an enzyme specifically adapted to act on *citric acid.* Both these enzymes are also found in the leaves of the cucumber. Finally, there may be separated from liver a *glucose dehydrogenase* which, in presence of methylene-blue, yields gluconic acid. All these last-mentioned dehydrogenases are *anaerobic*, that is to say, in contrast to the *aerobic* dehydrogenases, they act not in the presence of oxygen but only with methylene-blue or some other such acceptor.

Proline and **hydroxyproline** also are dehydrogenated by ground, dried liver. Nothing is yet known concerning the nature of the end-products.

2. RESPIRATION OF ACETIC BACTERIA WITH METHYLENE-BLUE

Obtaining the Bacteria.—Use either *B. pasteurianum* (bacteria of brewery acetic acid) or *B. orleanese* (bacteria of acetic acid from wine). Cultures of these can be obtained from the National Collection of Type Cultures, London, or the Institut für Gärungsgewerbe in Berlin. As a culture medium, use beer wort of specific gravity 1·0322. Deproteinise the wort of dark beer before use by heating for several hours in a steam steriliser, filter and dilute to the required specific gravity. Propagate in large conical flasks (capacity 2–3 litres) pouring 100 c.c. of wort into each and thus obtaining a large surface for the culture. Since the acetic bacteria are strongly aerobic a large surface is necessary in order to permit free acess of oxygen.

Flasks of the size mentioned yield 100–150 mg. of bacterial material (dry weight). For the experiment about to be described two flasks are ample. After pouring in the wort, close the flasks with plugs of cotton wool and sterilise twice for 1·5 to 2 hours, allowing 1 day to elapse between the sterilisations. Then inoculate with a few drops of the suspension of bacteria, taking care to maintain sterility, and incubate for 1 week at 28°–29°. Strong bacterial growth becomes apparent even after 2 days. Combine the contents of the flasks and spin them for 15 minutes in a high-speed centrifuge. Wash the deposited bacteria twice by stirring with physiological salt solution (9 g. of sodium chloride per litre of water) centrifuging each time, stir again with physiological salt solution and bring into suspension, pouring 10–15 c.c. into each flask. With a pipette, the required amount of suspension (the suspension contains about 10 mg. of bacteria per c.c.) can be withdrawn as desired. Preferably leave the suspension for a few days in a moderately cool room in order that the content of reserve material may decrease and hence so that the residual respiration may be small. Such bacterial suspensions maintain their maximal activity for 2 or 3 weeks. On no account must disinfectants, such as toluene or chloroform, be added. Always examine microscopically by Gram's colouring method in order to find out whether the material is free from yeast cells.[1]

Bertho's Methylene-blue Technique.[2]—*Apparatus required* (see Fig. 24): A number of test-tubes about 12 cm. long (capacity 20–25 c.c.) with side tube carrying a well-fitting glass stop-cock and a corresponding number of suitable two-holed rubber stoppers. Through one hole insert a glass tube having a right-angle bend and extending to the bottom of the tube. The shorter external arm of this tube carries a glass stop-cock. Through the other hole insert a dropping funnel with a glass stopper, the stem being bent at an angle of 60° and extending about 4 cm. into the test-tube. Place the fitted test-tubes, when ready for use, in a thermostat, inclining them at an angle of about 30° to the horizontal, and immersing them so far that the water covers the side tube which is parallel to the surface. Insert the test-tubes into notched brass-rings in a plate which is kept continuously moving during the experiment. By loosening a wing-nut, adjust this plate to any desired position. Connect the right-angle bends of the inlet tubes, by means of rubber tubing, to a gas distributor through which oxygen-free nitrogen can be passed.

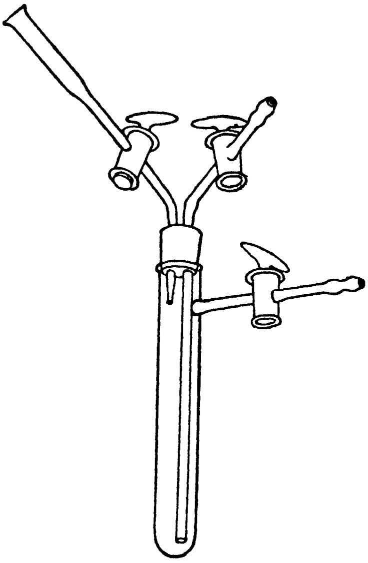


FIG. 24

Experiment.—Study the decolorisation of methylene-blue by "resting acetic bacteria", using six tubes. In each case use the same amount of bacteria (about 10 mg. dry weight per c.c.) and employ as donators: (1) methyl alcohol; (2) ethyl alcohol; (3)

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[1] For an introduction to the technique see Löhns, *Landwirtschaftlich-bakteriologisches Praktikum* (Berlin); Koch, *Bakteriologisches Praktikum* (Berlin).

[2] *Annalen*, 1929, 474, 8.

*iso*Propyl alcohol; (4) acetaldehyde; (5) ethyl alcohol in the presence of 0·002*N*-HCN; (6) a tube without donator for determination of residual reduction.

Procedure.—With a pipette, run the suspension of the bacteria, the buffer solution and the given amount of water into the funnels. Then close the test-tubes with the fitted stoppers. Turn the stopcocks to the " off " position, fit the tubes into the brass rings of the movable plate and make fast in the thermostat with screws. Temperature 28°–30°—this is optimal for acetic fermentation. Connect the inlet tubes by means of rubber tubing to the gas distributor and pass into the tubes successively oxygen-free nitrogen prepared by passing nitrogen over a copper spiral heated to a low red heat. In order to carry out this operation correctly, close all the stopcocks on the dropping funnels except that on the first tube. After 5 minutes—with gas passing at the rate of two bubbles per second—close the cock on the funnel and open that on the side tube so that the gas escapes through the latter. After 5 minutes close this cock, open that on the funnel of the second tube and proceed as before. The passing through of the nitrogen can be repeated in the same way but more rapidly. Now run the donators into the funnels from a pipette. In order to allow the various donators to run into the test-tubes, open the cocks of the funnels and then cautiously open those of the side tubes: close both sets of cocks immediately after the liquids have run in. Make sure that all but a very small amount of the donator runs in but on no account allow air to enter. Into tube 5 run at the same time the hydrocyanic acid prepared by diluting appropriately the commercial solution. Alternatively, simply use

Samples:

Tube No. . . .	1	2	3	4	5	6
Suspension of bacteria .	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
0·2*M*-Acetate buffer solution, p_H 5·6 . .	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
0·1*M*-Donator, (1 c.c. of each)	Methyl Alcohol	Ethyl Alcohol	*iso*Propyl Alcohol	Acetaldehyde	Ethyl Alcohol	—
0·02*M*-HCN . .	—	—	—	—	1 c.c.	—
Boiled-out, distilled water, c.c.	5·5	5·5	5·5	5·5	4·5	6·5
Methylene-blue solution (0·5 mg. per c.c.), in c.c. .	1	1	1	1	1	1
Boiled-out distilled water for washing out . .	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.

potassium cyanide solution although this may, in some circumstances, cause a shift of p_H because of the strongly alkaline reaction. Next run in the methylene-blue solution in the same way and finally wash with water, taking the same precautions. *Note the time at which the methylene-blue solution is added.* Then start the shaking at the rate of 140 strokes per minute (70 strokes in each direction). Decolorisation is complete when the shade of colour in the substrates is bluish-white. *Note the time when this shade is attained in each case.*

Compare the decolorisation times with the approximately inversely proportional manometric measurements of oxygen consumption in the experiment described on page 192. These times give a rather good idea of reducing power of the dehydrogenase of the acetic bacteria and, accordingly, of the corresponding intensity of oxidation (see p. 153).

Note that decolorisation is very slow in tube 1 since methyl alcohol is a very poor donator whilst, of the tubes 2, 3 and 4, tubes 2 and 4—since they contain respectively the natural substrate, ethyl alcohol, and an intermediate product of acetic fermentation, acetaldehyde—exhibit the shortest decolorisation periods. *iso*Propyl alcohol (tube 3) is likewise dehydrogenated with moderate intensity, but considerably more slowly, acetone being produced. Hydrocyanic acid (tube 5), although it acts, in respiration with oxygen, as a powerful poison towards the cells, does not increase the time of decolorisation which would indicate a decrease in the reducing power. The decolorisation time is rather decreased probably because methylene-blue forms a loose compound with the acid, and the compound has acceptor properties different from those of methylene-blue itself (von Euler). This lack of inhibitory action (on respiration with acceptors) of hydrocyanic acid will be discussed later (see p. 193). In tube 6 a small residual reduction, *i.e.* a relatively long decolorisation period is observed.

A brief *criticism* of the *methylene-blue technique* and of the use of methylene-blue as acceptor may be given in conclusion.[1]

(1) The method shows only the end-point of the reaction and not its kinetic course although in characterising the enzymes involved this course is significant. The determination of the period of decolorisation is a subjective process and hence liable to error.

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[1] For a manometric method of measuring the methylene-blue reaction see Reid, *Biochem. Z.*, 1931, **242**, 159.

(2) Both methylene-blue and its sparingly soluble leuco-compound have a detrimental adsorptive action. In high concentrations methylene-blue is poisonous to the cells. With this is connected the existence of a sparingly soluble nucleic acid salt (nucleus of the cell).

(3) The oxidation-reduction potential of methylene-blue (see p. 173) is very low in comparison with that of the cells.

(4) Since the oxidation-reduction potential of many of the systems present in the cells is higher than that of leucomethylene-blue, equilibria are occasionally set up in experiments with methylene-blue. In such cases the methylene-blue method is of limited value only.

The **ideal hydrogen acceptor** for decolorisation experiments must have the following properties :

(1) In order to prevent interference due to adsorption it should have a low molecular weight.

(2) Its oxidation-reduction potential must be in the neighbourhood of that of oxygen. Together with its hydrogenation product it must constitute a reversible system.

(3) It must be intensely coloured.

THE BACTERIAL DEHYDROGENASES

Amongst bacterial dehydrogenases that which has been most thoroughly studied is the dehydrogenase of *acetic bacteria*. As already mentioned, acetic fermentation was the first biological process in which the validity of the dehydrogenation theory was detected. Acetic fermentation has since been extensively studied (Wieland, Bertho [1]) for the purpose of throwing light on general questions concerning the theory of dehydrogenation, on the kinetics of the reaction when oxygen, methylene-blue and quinone are the acceptors and on the inhibitory action of cell poisons. It has been found that acetic fermentation consists of a series of dehydrogenation reactions in which *acetaldehyde* is an intermediate. A single dehydrogenase only is involved. Until the acetaldehyde stage is reached, oxygen alone acts as acceptor in the natural process [equation (I)]. The acetaldehyde can be transformed into *acetic acid* in *two* ways, *aerobically by dehydrogenation* (of its hydrate) by oxygen [equation

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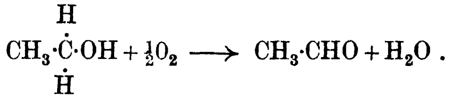
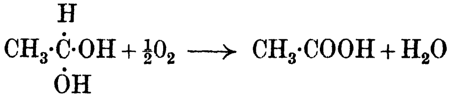
[1] *Annalen*, **1928**, **467**, 95 ; **1929**, **474**, 1 ; *Ergebn. Enzymforsch.*, **1932**, **1**, 231 (Leipzig).

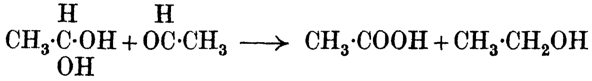
(II)] and *anaerobically by dismutation* [equation (III)] in which case a second molecule of acetaldehyde acts as acceptor



$$CH_3\cdot\underset{\dot{H}}{\overset{H}{\dot{C}}}\cdot OH + \tfrac{1}{2}O_2 \longrightarrow CH_3\cdot CHO + H_2O \quad . \quad . \quad . \qquad (I)$$



$$CH_3\cdot\underset{\dot{O}H}{\overset{H}{\dot{C}}}\cdot OH + \tfrac{1}{2}O_2 \longrightarrow CH_3\cdot COOH + H_2O \quad . \quad . \qquad (II)$$



$$CH_3\cdot\underset{OH}{\overset{H}{C}}\cdot OH + \overset{H}{OC}\cdot CH_3 \longrightarrow CH_3\cdot COOH + CH_3\cdot CH_2OH \quad . \qquad (III)$$

Acetic acid is produced mainly by the processes (I) and (II) in which oxygen is the acceptor whilst the dismutation of acetaldehyde (III) although it doubtless occurs (Neuberg, Simon, Windisch) is of less significance.[1] It is doubtful if the *dehydrogenase* and the *mutase* of acetic bacteria are altogether identical in view of results of experiments with preparations made with the use of acetone. But at least the aldehydemutase requires the co-operation of a *co-enzyme* which, however, is not identical with the co-enzyme of methylglyoxalase (cf. pp. 244 and 259). The latter co-enzyme is simply glutathione (Lohmann). However, for the aldehydrase of Schardinger's enzyme from milk (see p. 169) and for the enzyme of liver (see p. 160) the *fundamental rule of the dehydrogenation theory* holds, namely : *dehydrogenase = mutase.*

The dehydrogenase of the acetic bacteria, like bacterial dehydrogenases in general, is *group-specific* and accordingly acts on a great number of primary and secondary alcohols and aldehydes. This is quite clear from the decolorisation experiment described above. Primary alcohols, when so used, are only partially converted into acids. To a considerable extent the dehydrogenation proceeds only to the stage of the corresponding aldehyde which can easily be detected in the decolorised liquid by adding fuchsine-sulphurous acid. From *iso*propyl alcohol acetone is produced in a one-stage dehydrogenation (Bertho, Müller).

Extensive studies of the dehydrogenase systems of bacteria (*B. coli etc.*) have been made by Quastel, Haldane and their co-workers; all the factors involved in the respiration of cells being taken into account. In this connexion an investigation by Cook, Haldane and Mapson,[1] of the dehydrogenation of *succinic acid, lactic acid* and *formic acid* by *B. coli* treated with toluene is of interest. According to these authors three different dehydrogenases and as many (haemin-containing) oxidases (see p. 222) are concerned in this process. They conclude on the basis of the inhibition produced by certain sulphonic acids (including 8-hydroxyquinoline sulphonic acid, Eichhorn's reagent) which form complexes that *formico-dehydrogenase* contains copper. This is the first time that it has been shown to be probable that a dehydrogenase *contains a metal.*

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[1] **Bertho and Basu, *Annalen*, 1931, 485, 26.**

Of especial interest is the fact, already well known for long, that many bacteria (*e.g.* from the *Coli* group) activate *molecular hydrogen* and transfer it to acceptors. The *hydrogenase* concerned was isolated by Stephenson. It is sensitive to the action of hydrocyanic acid and hence behaves like an inorganic hydrogenation catalyst (see p. 140). Accordingly, it is really only a special type of dehydrogenase. On the other hand, a bacterial enzyme extensively characterised by Harrison, namely, *hydrogenylase,* is able to dehydrogenate organic substrates such as, say, *formic acid* with production of molecular hydrogen.

The oxidative degradation of *glucose* to carbon dioxide and water by *lactic acid bacteria* has been recognised to be a dehydrogenation process in which the oxygen can be replaced by quinone or methylene-blue. When lactic acid producers free from catalase, such as *B. Delbruckii* or *B. acidophilus,* are used the oxygen is recovered as hydrogen peroxide.[2] Thus the process is a perfectly clear example of dehydrogenation (see also p. 226). *Lactic acid* also is dehydrogenated by lactic acid bacteria with production of pyruvic acid (Simon).

3. RESPIRATION OF ACETIC BACTERIA WITH QUINONE[3]

Experiment.—Determine the consumption of quinone by acetic bacteria in presence of various donators, namely, the same as those used in the preceding experiment. Since quinol is not oxidised in acid media larger samples (about 100 c.c.) can be used in this case, complete exclusion of air is not necessary and samples can be withdrawn by means of a pipette from time to time—in the present case every 15 minutes—in order that the quinone still unconsumed may be volumetrically determined according to Valeur with potassium iodide and thiosulphate. Plot the amounts of quinone consumed on a curve against the times (abscissae) and thus demonstrate the kinetics of the respiratory action of quinone (as ordinates, c.c. of 0·01 N-quinone solution).

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[1] *Biochem. J.*, 1931, 25, 534.

[2] Bertho and Glück, *Naturwiss.*, 1931, 19, 88; *Annalen*, 1932, 494, 159.

[3] Wieland and Bertho, *Annalen*, 1928, 467, 109; Bertho, *Annalen*, 1929, 474, 33.

Samples :

Flask No.	1	2	3	4	5	6
Suspension of bacteria (1 mg. dry matter per c.c.)	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
0·02N-Acetate buffer solution, p_H 5·6	10 c.c.	10 c.c.	10 c.c.	10 c.c.	10 c.c.	10 c.c.
0·1M-Donator (10 c.c. of each)	Methyl Alcohol	Ethyl Alcohol	*iso*Propyl Alcohol	Acetaldehyde	Ethyl Alcohol	—
0·02N-Hydrocyanic acid	—	—	—	—	10 c.c.	—
Water (in c.c.)	69	69	69	69	59	79
0·01M-Quinone solution (1·08 g. per litre)	10 c.c.	10 c.c.	10 c.c.	10 c.c.	10 c.c.	10 c.c.

Procedure.—Using retort stands and clamps, suspend six long-necked flasks (capacity 250 c.c.) in a thermostat at 28°–30°, pour into them the liquids shown in the table, waiting until the flasks have acquired the temperature of the water in the thermostat before adding the bacteria (*start of the reaction*, note the time). Shake the flasks well. In order to remove oxygen which, however, competes only very slightly with the quinone for the activated hydrogen of the donator, fit the flasks with two-holed rubber stoppers carrying inlet tubes bent at a right angle and reaching down near to the surface of the liquid, and, in the other hole, short escape-tubes provided with stop-cocks. From a gas distributor pass nitrogen through the flasks. Raise the stoppers only when removing samples (10 c.c.), withdrawing them at intervals of about 15 minutes. Keep the bacteria in suspension by repeated shaking. Transfer the samples (taken with a pipette) for the determination to conical flasks, add 20 c.c. portions of 2 N-sulphuric acid (*end of the reaction*, note the time) and 5 c.c. portions of potassium iodide solution (5 per cent), leave for 25–30 minutes to allow for complete separation of iodine, keeping the flasks closed, and titrate from a micro-burette with 0·1 N-thiosulphate, using starch as indicator.

For each sample of the original mixture calculate the corresponding volumes of 0·01 N-quinone solution and plot as ordinates against the times of withdrawal of the samples for determination as abscissae. The curves obtained, which are straight lines only with the higher concentrations of quinone (optimal concentration of the acceptor), approach the X-axis asymptotically. Here also, as with methylene-blue the primary alcohols undergo only partial dehydrogenation to the acid. The aldehyde which is produced can be detected with fuchsine-sulphurous acid. In order to determine how much acetic acid has been produced, titrate sample 2 with 0·1 N-NaOH solution from a micro-burette, using bromothymolphthalein as indicator. Then, from the equations on p. 165 it is easy to find what percentage of the quinone added is used for the stage acetaldehyde-acetic acid.

The action of the acceptor, quinone, in the six samples expresses itself in a way very similar to that expressed by methylene-blue in the preceding experiment. In sample 1 where methyl alcohol is the donator there is only very slight reaction whilst the greatest transformations occur with ethyl alcohol (sample 2) and acetaldehyde (sample 4). *iso*-Propyl alcohol (sample 3) is likewise dehydrogenated to a large extent. Hydrocyanic acid (sample 5), as is generally the case in respiration with acceptors, has very little effect here also. The residual respiration is determined in sample 6. After a short time the action ceases because the donators of the cell itself are used up. Accordingly, constant values are obtained in the subsequent titrations of this sample.

Quinone cannot be used as acceptor with success in all cases. The reasons for this are to be found partly in the tanning action of quinone and partly also in its high oxidation-reduction potential. Methylene-blue also, because of its power to precipitate proteins is frequently limited in its application with enzyme solutions. When the *extents of transformation with quinone and methylene-blue* are considered, it is found that in the cases just discussed there is an extraordinarily great degree of difference between the two. The acceptor action of methylene-blue although very noticeable because of the strong colour of the dye is, in this case, of a lower order than that of quinone. In experiments with cell material it is often so and depends on the peculiarities, structure and mechanism of action of the cell. But this need not be the explanation in every case and experimental evidence of the fact will be advanced later (see p. 195), the extent of consumption of oxygen being taken into account for comparison. In order to exclude from the start any false conception, it must be mentioned here that just as the *rate* of reaction has nothing to do with the *affinity*, so in dehydrogenation reactions the magnitude of the *oxidation-reduction potential*, which is, in fact, a measure of the amount of free energy of the system undergoing dehydrogenation, has, in general, no connexion with the reaction velocity. This depends on the molecular structure and on the catalysts which are present. According to Dimroth,[1] however, there exists in quinones, chemically very closely related (" dynamic homologues "), parallelism between reaction velocity and affinity.

4. Preparation of Material containing Schardinger's Enzyme [2]

Transfer cows' milk (obtained, if possible, by milking under sterile conditions) to a flask containing a little thymol in toluene immediately after milking and shake. In this condition the milk can be kept in the refrigerator for at least three weeks without undergoing the slightest change in its enzymic action. The absolute amount of enzyme in the milk varies a little, depending on the diet and probably still more on the age of the cow. The length of the interval since the last calving before the milking seems to be the most important factor. Variations of about ±50 per cent from an average are encountered.

A description is given below of the *preparation of the enzyme from cream* which has a high content of the enzyme. Cream is chosen because it is as far as possible free from casein. The proportion of enzyme in the skim-milk is neglected.

Remove the skim-milk and prepare very highly concentrated cream having almost the consistency of butter by using a separator turning at 4000 revolutions per minute. Spread out the cream in a thin layer in a shallow dish and dry over concentrated sulphuric acid and much solid potassium hydroxide. first at the reduced pressure produced by a water-jet pump and then at that produced by an oil pump. Extract exhaustively the material thus obtained (it resembles butter fat) first with purest absolute ether in the thimble of a Soxhlet apparatus and then with light petroleum (boiling point, 25°–38°) which has been distilled over sodium. The residual powder is very active and can be preserved without loss of activity. In the presence of phosphate buffer solution of p_H 8·0 the material dissolves in water at 37° to give a slightly opalescent liquid having a few flocculent particles of casein in suspension. Almost complete clarification without much loss of activity is attained by shaking the solution with a little aluminium hydroxide C.[1] The clarified solution prepared as required from the powder can be used for such experiments as it is desired to carry out. The powder is best kept under low-boiling (25°–38°) light petroleum which has been distilled over sodium. Powder removed for experiment is lifted out on a nickel spatula and spread on a sheet of filter paper so that, in quite a short time, the petroleum evaporates. Material obtained in this way is about 100 times as active as the corresponding amount of milk (dry-matter basis).

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[1] *Angew. Chem.*, 1933, 46, 571.

[2] Wieland and Rosenfeld, *Annalen*, 1929, 477, 32; Wieland and Macrae, *Annalen*, 1930, 483, 217.

Schardinger found, in 1902, that raw milk can decolorise methylene-blue in presence of formaldehyde, and Wieland showed in 1914 that an active *dehydrogenase* which is able in co-operation with either methylene-blue or oxygen to convert acetaldehyde or its hydrate (see p. 165) into acid is involved. Finally, he showed that in complete harmony with the dehydrogenation theory this dehydrogenase could act as a mutase, dismuting aldehyde into acid and alcohol. Morgan, Stewart and Hopkins showed that the enzyme of milk can also dehydrogenate hypoxanthine and xanthine with co-operation of oxygen, methylene-blue or nitrates (Bach) yielding uric acid. Dixon and Thurlow[2] were the first to obtain the enzyme in solid form and they were also the first to show that the oxygen consumed in the dehydrogenation of hypoxanthine and xanthine reappears as *hydrogen peroxide.* Accordingly, the transformation of hypoxanthine and xanthine into uric acid by way of the hydrates as intermediates proceeds as follows :

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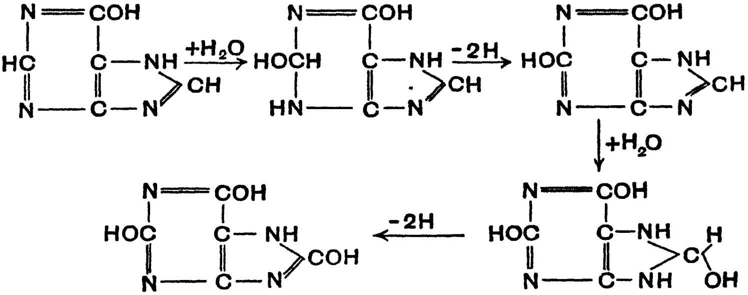
[1] Willstätter and Kraut, *Ber.*, 1923, 56 [B], 1117.

[2] *Biochem. J.*, 1924, 18, 971, 976, 989.



It has not yet been finally decided whether the dehydrogenation of aldehydes and of purines in milk is accelerated by *one* enzyme or whether two different enzymes, an *aldehydrase* and a *xanthine dehydrogenase* are involved. According to Wieland and Rosenfeld, who have made comparative measurements of the activity of one and the same enzyme solution towards the two groups of hydrogen donators, the results obtained can hardly be explained except on the supposition that two different enzymes are present. It has been found, for example, that the ratio of the times of decolorisation of methylene-blue with xanthine and salicylaldehyde is not constant for various sorts of milk and so on.

In order to be able to compare the actions of the various enzymes it is useful to introduce *units* of measurement (*enzyme units*) as has been done in other cases by Willstätter.

An xanthine dehydrogenase unit is that amount of the enzyme which, in the presence of 0·2 *c.c. of* 0·01*M-xanthine solution at* p_H 8, *the total volume being* 5 *c.c., decolorises* 1 *c.c. of* 0·001 *N-methylene-blue solution at* 37° *in* 5 *minutes.*

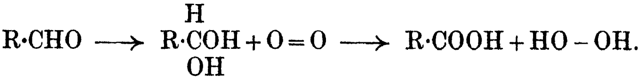
A salicylaldehydrase unit is that amount of enzyme which, in the presence of 0·2 *c.c. of* 0·02*M-salicylaldehyde solution at* p_H 8, *the total volume being* 5 *c.c., decolorises* 1 *c.c. of* 0·001*N-methylene-blue solution at* 37° *in* 5 *minutes* (Wieland and Rosenfeld).

As Dixon and Thurlow have already shown the time of decolorisation under the above conditions is inversely proportional to the amount of enzyme. In both cases the concentration of the substrate is optimal. In order that exact comparison may be possible it is essential that this be so (enzyme-time law, see p. 157).

Whilst the aldehydrase content of milk is rather constant, the xanthine dehydrogenase content is subject to very considerable variations. The ratio xanthine dehydrogenase : aldehydrase is shifted so as to favour the former when the milk is separated into cream and skim-milk apparently because cream exhibits a greater power to adsorb xanthine dehydrogenase.

It is noteworthy that when milk is left to stand, the xanthine dehydrogenase action increases quite considerably, while the aldehydrase action remains almost unchanged. A litre of cows' milk contains about 2000–2500 xanthine dehydrogenase units and about 800–1000 salicylaldehydrase units.

Dixon, who prepared the first active sample of the enzyme, showed that oxygen has a detrimental effect on the reaction with purines. It was found that the enzyme was destroyed only during and as a result of its dehydrogenating activity. The cause of the damage is the *hydrogen peroxide*, which is produced in accordance with the dehydrogenation theory. The yield, in the case of the purines is 100 per cent of the calculated amount. Hence, as Dixon first showed, the enzyme is not destroyed if *catalase* (which, as is well known, destroys hydrogen peroxide) is added (see p. 201). The function of *catalase as a protective enzyme* is evident here. The production of hydrogen peroxide can be demonstrated in various ways.[1] The most striking of these is to use a cerium salt which is converted into the reddish-brown cerium peroxide (tetravalent cerium) (Wieland and Rosenfeld). Hydrogen peroxide is also found when aldehydes undergo enzymic oxidation :



$$\mathrm{R{\cdot}CHO} \longrightarrow \mathrm{R{\cdot}\overset{H}{\underset{OH}{C}}OH + O{=}O} \longrightarrow \mathrm{R{\cdot}COOH + HO{-}OH}.$$

Here, no matter whether acetaldehyde, formaldehyde, benzaldehyde, salicylaldehyde or anisaldehyde be used, 75–90 per cent of the amount of hydrogen peroxide (calculated according to the above equation) can be detected when cerium salt is used.

It is worthy of note that the action of the Schardinger enzyme (or its component enzymes) is but slightly inhibited by *hydrocyanic acid*. Apparently a specific surface-action (expulsion) is involved (see p. 197).

The enzymic dismutation of aldehydes by the aldehydrase of milk has already been discussed above.

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[1] Schales, *Ber.*, 1938, 71 [B], 447.

OXIDATION-REDUCTION POTENTIAL

THEORY

It was pointed out on p. 142 that in order to determine affinity values exactly the oxidation-reduction potential of a system consisting of a mixture of the non-hydrogenated (unreduced) and hydrogenated (reduced) forms of a substance (such as methylene-blue and leucomethylene-blue) must be measured. When, as in this particular case, the system is reversible the potential determined is a direct measure of the free energy. This does not hold for irreversible systems—and irreversible systems greatly predominate in nature. Nevertheless, here also, measurement of the potential provides valuable information.

Oxidation-reduction potentials of acceptors related to components of cells, of foreign acceptors and of their hydrogenation products have been determined especially by Clark, Dixon, Conant and Biilmann. Their views about the nature of the processes concerned are different but lead eventually to the same conclusion. In what follows, Dixon's interpretation[1] is outlined because it harmonises with Wieland's conception of hydrogenation and dehydrogenation.

The **hydrogen electrode** (see p. 23) is the basis of the interpretation. The action of this electrode involves an equilibrium between the H-ions of the aqueous solution and the activated hydrogen atoms at the surface of the platinum, this equilibrium being itself dependent on the partial pressure of the hydrogen in the gas space. For the hydrogen electrode, the following expression, simply derived from Nernst's general electrode formula, holds :

$$E_H = \frac{RT}{F} \log \frac{[H^\cdot]}{\sqrt{P}},$$

where $[H^\cdot]$ is the hydrogen-ion concentration and P is the pressure of the hydrogen gas. Since, by definition, the hydrogen electrode is a null-point instrument, it follows that $E_H = 0$ and $[H^\cdot]$ and $P = 1$. Such an electrode serves as a reference electrode.

As was pointed out on page 145 a dissolved substance capable of being dehydrogenated (oxidised) will, in presence of platinum or palladium, give up its hydrogen to the metal and accordingly will be oxidised if the *dehydrogenation* process is exothermic. Conversely a substance capable of being hydrogenated (reduced) will take hydrogen from platinum or palladium and will be reduced if the *hydrogenation* reaction is exothermic. In each case an equilibrium is attained which, in the most favourable case, is a genuine equilibrium. Cases of the first kind are known, it is true, but are of small importance. Cases of the second kind are those of reducible dyes such as methylene-blue and indigo, quinone and so forth. Now these are just the substances which exhibit oxidation-reduction potential. Such potentials are set up when solutions of the substances mentioned are used as media in conjunction with a platinum electrode. In all cases the processes concerned are identical and the *relations* between *hydrogenation* and *dehydrogenation* in Wieland's sense on the one hand, and the phenomena of oxidation-reduction potential on the other, are established.

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[1] *Proc. Roy. Soc.*, 1927, 101 B, 57.

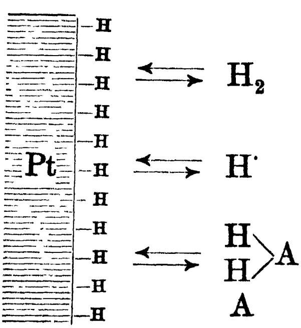

FIG. 25

With the two *equilibria* [(I), (II)] (see Fig. 25) which are also concerned in the action of the hydrogen electrode, there is associated, in this case, a third (III).

The active hydrogen of the electrode is in equilibrium with the partial pressure of the hydrogen :

$$2H \rightleftharpoons H_2 \quad . \quad . \quad . \quad . \quad (I)$$

But the active hydrogen is also in equilibrium with the H-ions of the solution :

$$H - \ominus \rightleftharpoons H^{\cdot} \quad . \quad . \quad . \quad . \quad (II)$$

In the case under consideration, however, when the oxidation or reduction stage of a system is present a further equilibrium is attained, namely, the equilibrium :

$$AH_2 \rightleftharpoons A + 2H \quad . \quad . \quad . \quad . \quad (III)$$

where H signifies the activated hydrogen at the surface of the platinum. Equation (III) holds for the special case where a substance is used, the hydrogenation product of which is richer by two atoms of hydrogen than the dehydrogenation product. Hence equilibrium

(III) also determines the position of equilibria (I) and (II) (see scheme). It is easily seen that a ratio first chosen determines equilibrium (III) and hence also affects (I) and (II).

$$\frac{\text{Reduction stage}}{\text{Oxidation stage}} = \frac{\mathrm{AH_2}}{\mathrm{A}},$$

Accordingly, the electrode behaves like a hydrogen electrode which is in equilibrium with a hydrogen atmosphere of exceptionally low pressure. It follows that the equation for the hydrogen electrode :

$$E_h = \frac{RT}{2F} \log \frac{1}{P} - \frac{RT}{F} \cdot p_{\mathrm{H}},$$

suitably altered may be applied. Finally we obtain :

$$E_h = E_{0\ \text{const.}} - \frac{RT}{2F} \log \frac{\mathrm{AH_2}}{\mathrm{A}} - \frac{RT}{F} \cdot p_{\mathrm{H}}.$$

This general *equation for oxidation-reduction potential* holds in this simple form for systems which are not ionised—say for the ethane-ethylene electrode (if this could be brought into equilibrium) and for the *quinone-quinol electrode* (in a high degree of approximation) in strongly *acid* solution where ionisation is very slight (see p. 25).

AH_2 is the *undissociated* stage of the reduction. If dissociation of the type $AH^{-} + H^{\cdot}$ or $A^{--} + 2H^{\cdot}$ occurs (dye in the reduced condition, quinol, *etc.*), account must be taken of this in the formula. The ionic equilibria and the constants appear in the equation. For example, in the case of the *quinone-quinol electrode* the following expression is obtained :

$$E_0 - \frac{RT}{2F} \log \frac{\mathrm{AH_2}}{\mathrm{A}} + \frac{RT}{2F} \log \left(\mathrm{k_1 k_2} + \mathrm{k_1}[\mathrm{H}^{\cdot}] + [\mathrm{H}^{\cdot}]^2\right).$$

This is the expression obtained by Clark[1] as well as by Dixon and Biilmann, although Clark's conception depends on the theory of the transfer of electrons, and Dixon regards atomic hydrogen, in accordance with Wieland's theory, as the chief agent involved—the result is, and must be, the same.

Consider first the *electrode equation* for a system which has an oxidation-reduction potential. According to this equation, the E.M.F. of a system depends on the magnitude of the constant E_0, the ratio stage of reduction : stage of oxidation, and the p_H of the medium. If $\frac{AH_2}{A}$ is kept constant in a series of experiments then

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[1] See Clark, *The Determination of Hydrogen Ions* (Baltimore, 1925); Michaelis, *Oxidation-Reduction Potentials* (Philadelphia, 1930).

$$E = E_0' - \frac{RT}{F} \cdot p_H,$$

namely, the equation for the hydrogen electrode. When there are two such half-elements with different p_H of which one is known, say, as in the case of two *quinhydrone electrodes* which are connected in series, the unknown p_H of the other half-element can be measured. This follows from the equation :

$$E = \frac{RT}{F} \log \frac{h_1}{h_2},$$

where h_1 is the hydrogen-ion concentration of the known and h_2 is that of the unknown solution.

If, on the other hand, the p_H is kept constant, the equation takes the form :

$$E = E_0'' - \frac{RT}{2F} \log \frac{\text{Red.}}{\text{Ox.}},$$

where $\frac{\text{Red.}}{\text{Ox.}}$ is written for $\frac{AH_2}{A}$. E_0'' is the same for one and the same p_H and is not dependent on the ratio Red. : Ox. When this ratio is equal to 1 then $E = E_0''$. *This constant is characteristic for each individual oxidation-reduction system and has a fixed value for each* p_H. In Fig. 26, relations between E_h and p_H are shown in graphical form. It is clear that a system with a high oxidation-reduction potential must reduce one with a lower oxidation-reduction potential. Thus leucomethylene-blue can be titrated by quinone which is thereby reduced. $TiCl_3$ on the other hand reduces methylene-blue.

Thus, by measuring the E.M.F. of such notably *reversible* systems, numerical relations between oxidation and reduction processes are obtained and at the same time a direct measure of the affinities is made. The values for the heats of reaction calculated from thermochemical data, on the other hand, are only approximate measures. From the E.M.F. of a reversible process the corresponding calorie value can easily be calculated by means of a simple equation. The value for *maximal work* is thus obtained :

$$A_m = n_eFE = n_e 23050 E \text{ cal. } (n_e = \text{equivalents}).$$

Exact relations, when the ratio Red. : Ox. *varies*, are shown in Fig. 27 which is also based on the electrode formula.

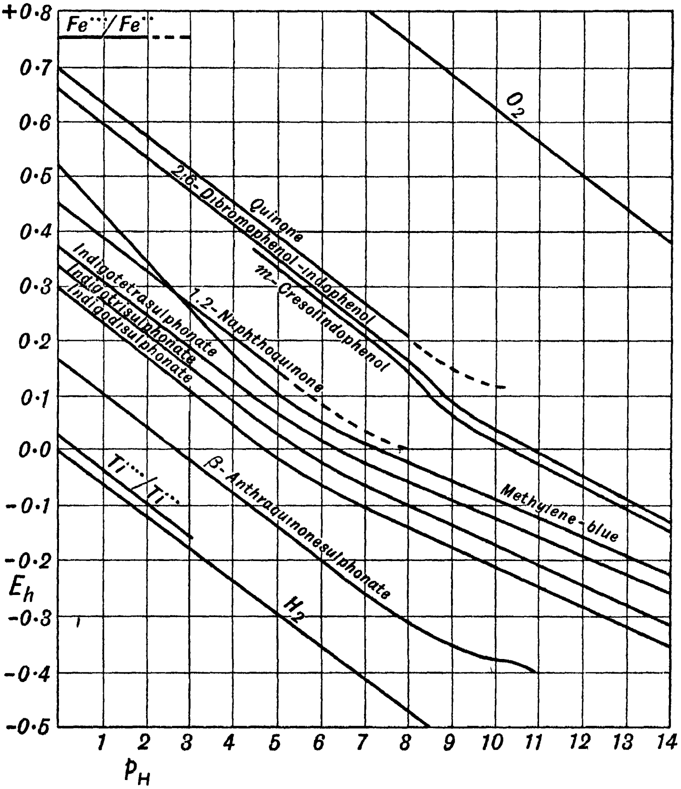

FIG. 26

In this Fig., percentage reductions or oxidations are the abscissae and E.M.F. are the ordinates. The various curves hold for one and the same system at various p_H, for various systems at the same p_H, or for various systems at various p_H. Curves with different n-values (n = equivalents) have different forms.

The points of intersection of the curves with the 50 per cent line (which corresponds to the Red. : Ox. ratio equal to 1) gives, by definition, the constant E_0-values which, for a particular system at a particular p_H, are independent of the ratio $\frac{AH_2}{A}$. The total concentration $AH_2 + A$ has no effect on E_h. But it has also been found that a variable ratio $\frac{AH_2}{H}$ has only slight effect within narrow limits on E_h.

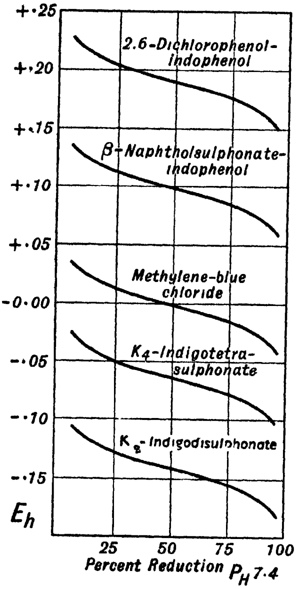

FIG. 27

Analogous to these curves are the titration curves of acids. In the latter, the p_H of the liquid which is titrated corresponds to E_h and the E.M.F. corresponds to the H_2-electrode used in the titration. Here the abcissae are the percentages of neutralisation ; there they are the percentages of reduction. The flat, middle portions of the curves are characteristic of *buffer action* and, in the oxidation-reduction potential curves the corresponding flat, middle portions are characteristic of an effect which Clark has called " poising action ", that is to say, that in these parts of the curves small changes in the ratio Red. : Ox. have no appreciable effect on the E.M.F. From this it follows that the addition of a large amount of one oxidation-reduction system to a small amount of another acts like a buffer solution on the p_H. *The oxidation-reduction system which is in excess imposes its potential on the other.*

On this depends the so-called *oxidation-reduction indicator method,* a method of determining oxidation-reduction potentials by means of indicators. The procedure consists of adding *very small amounts* of dyes of known oxidation-reduction potential (Clark, Conant) to the system to be measured.

When substances which are in oxidation-reduction potential equilibrium have different colours according as they are oxidised or reduced, the change of colour which takes place in the neighbourhood of their normal potential serves as a means of determining the *oxidation-reduction potential of a medium* provided that the substances are present in concentration low enough not to alter the equilibrium in the medium. The method corresponds to a large extent with that used for determining p_H with indicators. Thus, if a dye is reduced (decolorised), then its oxidation-reduction potential is greater than that of the unknown system. If half of it is reduced, then the oxidation-reduction potentials are equal; if 99 per cent of it is reduced, then the potential of the dye is about 60 millivolts greater. Clark and his co-workers prepared a scale of dyes for this purpose and various other authors have subsequently extended this scale (see p. 183). The next experiment described (see p. 184), concerns the measurement of an oxidation-reduction potential in this way.

This *indicator method* may be compared with the *method of measuring the potential* which has been described. In both methods the r_H values are determined. This value, again, has its analogue, namely, p_H

$$p_H = -\log [H^{\cdot}],\ r_H = -\log P,$$

where P is the pressure of the hydrogen on the platinum electrode of a system which exhibits oxidation-reduction potential. This pressure, of course, varies from case to case. The greater P is, the more does the potential approach that of the hydrogen electrode, for which, since $P=1$, $r_H=0$. Accordingly, a low r_H-value corresponds to a strong tendency to reduce. The system methylene-blue-leucomethylene-blue has a low r_H. Hence it reduces quinone to quinol which two substances form a system having a higher r_H. Accordingly, leucomethylene-blue can be titrated with quinone. The oxygen electrode has $r_H=41$.

BIOLOGICAL OXIDATION-REDUCTION SYSTEMS

In the introductory chapter only reversible systems, which can at once be symbolised by means of the electrode equation, were considered. The biologically important oxidation-reduction systems which occur in the cells are, for the most part, *irreversible*. This holds for the SS-SH systems, such as cystine-cysteine, and oxidised glutathione-reduced glutathione which were studied by Dixon, Quastel and Clark. Conant has investigated a series of irreversible systems composed of pure organic compounds. The potential of an irreversible system is by no means a measure of the affinities involved. Hence if it is desired to obtain information as to whether an irreversible system can take part in a hydrogenation or dehydrogenation the thermochemical data must be determined, this being the only procedure available. Nevertheless, very valuable results are obtained from measurements of potentials of systems in which irreversible systems play a part but are accompanied by an acceptor, say methylene-blue, in large amount, which brings the systems to its r_H. These results throw light on enzyme action and on the kinetics of enzymic oxidation-reduction (see below).

There is still a great deal to be explained in connexion with the establishment of irreversible potentials. When the potential of a solution of an SH-compound containing H-ions is followed potentiometrically it is found that, with time, the potential becomes more and more negative and finally, at a certain stage, becomes constant. With reversible systems, on the other hand, the potentials very rapidly disappear. Dixon[1] has attempted to explain this behaviour of the electrodes and has deduced an electrode equation based on kinetic considerations.

The curves of potential, which are obtained when a suspension in buffered solution of *washed or washed and boiled cells*, such as those of yeast, bacteria or muscle is used as the medium for an electrode, indicate the presence of irreversible systems in the cells. And actually cells do contain such systems. The most important are those containing sulphur (glutathione) which play an important part in various ways in the cells[2] (see pp. 116, 260). In part, these systems are to be included amongst the thermostable donators of Hopkins (see p. 157). Palladin's *chromogens*[3] (respiratory pigments) probably also play a significant part amongst these systems.

Clark and his co-workers have also shown that a platinum or gold electrode in *washed yeast cells* acquires a reproducible potential and that the addition of *succinic acid* makes the potential more negative. This indicates the dehydrogenating action of the cells. Similar experiments can be carried out with Schardinger's enzyme.

The *measurement of the potential of suspensions of cells* is an important procedure for biologists because cell-material can be characterised by its oxidation-reduction potential.

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[1] *Proc. Roy. Soc.*, 1927, 101 B, 57.

[2] For further discussion of this point see Bersin, *Ergebn. Enzymforsch.*, 1935, 4, 68.

[3] *Z. physiol. Chem.*, 1908, 55, 207.

When the measurements are made under aerobic conditions it is found that the oxidation-reduction potential of the cell is, on the average, 0·1–0·2 volt (p_H 7) that is, it lies almost in the zone of 2 : 6-dibromoindophenol.

In order to determine the potential in living cells, *small amounts of oxidation-reduction indicators may be injected* (" micro-injection " procedure) as an alternative to the potentiometric processes mentioned above. Investigations have been made in this way by J. and D. Needham, Wurmser and others. A value of +0·2 volt, corresponding to an r_H-value of 20, is obtained for aerated cells. This value agrees with that determined electrometrically. One of the main questions to be decided is whether or not *genuine reversible oxidation-reduction equilibria* also exist in living cells. The presence of oxygen, which may take part in the oxidation-reduction system of the cells, complicates the decision on this question. Wurmser and Geloso,[1] after studying oxidation-reduction systems derived from glucose, came to the conclusion that such reversible systems occur in the cells, that they are independent of each other, and that, in the syntheses which occur in living cells (these syntheses, in fact, are chiefly reductions), they serve as hydrogen donators.

Those investigations which are of greatest importance to the biochemist, namely, *measurements of oxidation-reduction potential of suspensions of cells in the presence of acceptors* and, in addition, *measurements of oxidation-reduction potential of defined donator-enzyme-acceptor systems* yield kinetic data which the Thunberg methylene-blue method, because of its quantitative-qualitative character, cannot yield.

When yeast cells are brought into contact with methylene-blue, the latter, because of the donator content and the SH-systems, etc., of the cells, is decolorised. The potential of the mixture—which must be measured, of course, in a vacuum or in the absence of oxygen; say in an atmosphere of nitrogen—decreases. Towards the cells the system methylene-blue-leucomethylene-blue exhibits the poising effect and accordingly determines the r_H. The curve of the potential is almost quite identical with the titration curve of methylene-blue with titanium trichloride at the same p_H. From the curve the amounts of reduced methylene-blue for the various E_h-values can be obtained. It is found that the reaction is unimolecular. Hence, by means of *measurements of potential* the

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[1] *J. Chim. phys.*, **1928, 25, 641; 1929, 26, 424, 447.**

existence of dehydrogenases can be detected, on the one hand, and the *kinetics* of acceptor-reactions, on the other, can be followed. Without doubt, the method outlined is the sole method suitable for following the consumption of the acceptor when dealing with substrates which contain much tissue where the volumetric determination of methylene-blue cannot be carried out, if for no other reason than that the tissue adsorbs methylene-blue.

From what has been said it is clear that the most important objection which can be made in connexion with the use of acceptors is that the behaviour of foreign acceptors is not directly comparable with that of oxygen (which is not foreign to the cells) because the two systems have different r_H; that is to say, they exhibit the poising effect (which, moreover, is artificially imposed on the cells when an acceptor is used) in different regions. In other words, it may be asked, what is the relation, if any, between the oxygen-consuming aerobic processes and the results of the acceptor method. This is one of the chief objections brought by Clark against Wieland's theory in so far as it aims at explaining what processes actually occur in the cells and does not merely offer a formal scheme. Since there is no complete substitute for oxygen as acceptor (see p. 164) no exact refutation of this objection can be provided. But beyond doubt general statements concerning respiration with oxygen, based on the use of acceptor systems of different r_H, can be made. It has been found that the r_H, for example, is not of peculiar significance for the acceptor reaction.

Up to the present it has been possible, in a few individual cases only, to demonstrate, by the *electrometric procedure,* the existence of a thermodynamically reversible equilibrium *in a donator-dehydrogenase system.* The reasons for this negative result are of very many kinds. For example, the potential value determined may be derived from an equilibrium set up between an acceptor system present in the enzyme solution and the electrode. That method in which the affinities involved in a biological dehydrogenation reaction are determined by means of *oxidation-reduction indicators* must then be employed. It may be assumed that when an enzyme-substrate system decolorises a series of indicators which have nearly related chemical constitutions whilst those oxidation-reduction indicators of the same class but having lower oxidation-reduction potential are not decolorised, then the system under investigation has an oxidation-reduction potential lying between the lowest potential value of the former and the highest of the latter. Difficulties should arise only when the enzyme concerned, because of its pronounced specificity towards acceptors (see p. 196), cannot be appropriately used with most of the dyes of the scale of indicators. The rate at which the equilibrium is attained is immaterial and only the final value need be considered. Thunberg and also Quastel and Whetham have determined in this way the normal potential of the equilibrium mixture *succinic acid-succino-dehydrogenase-fumaric acid* (see p. 156). The succino-dehydrogenase was obtained from muscle or bacteria. The difficulty caused by the presence of fumarase can be overcome by adding, at the beginning, a certain amount of malic acid, into which fumaric acid is partly converted by the action of fumarase or else by destroying the fumarase by heating for 30 minutes at 50° as suggested by Alwall.

	E_h at p_H 7 (volt)	r_H
Hydrogen electrode	−0·421	0·0
Neutral-red iodide (approximate)	−0·30	4·0
K_2 Indigodisulphonate (indigo carmine)	−0·125	9·9
K_3 Indigotrisulphonate	−0·081	11·3
K_4 Indigotetrasulphonate	−0·046	12·5
Methylene-blue chloride	+0·011	14·4 (slightly poisonous)
1-Naphthol-2-sulphonate-indophenol	0·119	18·0
o-Cresol-indo-2 : 6-dichlorophenol	0·181	20·1
o-Cresol-indophenol	0·195	20·5
Phenol-indo-2 : 6-dibromophenol	0·217	21·3
Phenol-blue chloride	0·227	21·6
o-Chlorophenol-indophenol	0·233	21·8
m-Bromophenol-indophenol	0·248	22·3
Phenol-*m*-sulphonate-indo-2 : 6-dibromophenol	0·273	23·1
Oxygen electrode	0·81	41·0

This equilibrium was also determined *potentiometrically* by Lehmann[1] after he also had excluded the action of fumarase. This was the first time that such an equilibrium had been determined potentiometrically. The value obtained at p_H 7 and 37° was 0·4304 volt.

In the experiment next described below, those oxidation-reduction indicators are selected from a series, which are suitable for use with the reversible system *lactic acid-lactico-dehydrogenase-pyruvic acid.*

In the above table the oxidation-reduction potentials of a series of the most useful oxidation-reduction indicators are listed. The values given are relative to the potential of a hydrogen electrode having a hydrogen pressure of one atmosphere at p_H 0. This potential at p_H 7 has the value − 0·421 volt.

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[1] *Skand. Arch. Physiol.*, 1930, 58, 173.

5. OXIDATION-REDUCTION INDICATOR METHODS. OXIDATION OF LACTIC ACID BY CARDIAC MUSCLE

According to Szent-Györgyi and Banga[1] *lactic acid* is reversibly oxidised to *pyruvic acid* by a *dehydrogenase of cardiac muscle* which is activated by a *co-enzyme.* This co-enzyme is an adenylic acid which is not identical with the adenylic acid of muscle but is possibly identical with cozymase (see p. 242). The same dehydrogenation system converts *β-hydroxybutyric acid into β-ketobutyric acid.* Lactic acid, pyruvic acid, dehydrogenase and co-enzyme form a reversible oxidation-reduction system for which $E_0 = -0{\cdot}181$ volt. In order to determine the magnitude of this potential we use various oxidation-reduction indicators.

The potential of the system cardiac muscle-lactic acid-co-enzyme is *very strongly negative.* It decolorises methylene-blue at once as well as the much more negative *indigo carmine.* Janus green and its first reduction product *Janus red* and *neutral-red* are suitable for the purpose in view.

The *reversibility* of the system is established by the following experiments. On the one hand, lactic acid alone in the presence of enzyme (muscle) and co-enzyme decolorises Janus red and neutral-red, only incompletely. This shows, in the first place, that the oxidation-reduction potential of the system lies in the region of those of the two indicators. Since now, when lactic acid *and* pyruvic acid are present together the dyes are reduced to a much smaller extent than by lactic acid alone and since it is evident that the ratio $\frac{\text{Red.}}{\text{Ox.}}$ (see p. 176) plays a part in the process, the system must be reversible. The reversibility of the system:

$$\text{Dehydrogenase-Co-enzyme-}\begin{cases}\text{Lactic Acid}\\ \text{Pyruvic Acid}\end{cases} \rightleftharpoons \begin{matrix}\text{Dye}\\ \text{Leuco-Compound}\end{matrix}$$

is demonstrated especially clearly by the fact that in the presence of lactic acid and the system, the leuco-compounds are not oxidised;

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[1] *Z. physiol. Chem.*, **1933, 217, 43.**

but are oxidised especially strongly in presence of pyruvic acid and less strongly oxidised when lactic acid is present in addition. The equilibria which are attained with the dye and the leuco-compound are to a large extent identical. On the potentiometric measurement of the system, see *Z. physiol. Chem.*, 1933, **217**, 46.

Janus red is prepared by reducing (with hydrosulphite) Janus green to the leuco-dye which is then oxidised, but only to Janus red, by means of a current of air.

Procedure.—Use washed cardiac-muscle from the pig. As soon as possible after the animal is slaughtered pass the heart through a mincing machine, suspend the pulp so obtained in twenty volumes of distilled water, leave for 10 minutes, with stirring, filter through cloth, squeeze, wash again and squeeze a second time.

(1) Place in each of nine Thunberg tubes 0·5 g. of washed cardiac muscle, 2 c.c. of phosphate buffer solution ($0{\cdot}067M$, p_H 7) and 1 c.c. of 0·02 per cent solution of Janus red. Use tubes which have cylindrical cups fused in at the bottom. Pour into the cups of each of three of the tubes 1 c.c. of $0{\cdot}2M$-sodium lactate solution and 1 c.c. of water, into the cups of each of three others 1 c.c. of $0{\cdot}2M$-sodium pyruvate solution and 1 c.c. of water and into the cups of each of the remaining tubes 1 c.c. of $0{\cdot}2M$-sodium lactate solution and 1 c.c. of $0{\cdot}2M$-sodium pyruvate solution. Thoroughly evacuate all the tubes as described on p. 155, pour the solutions in the cups into the mixture of muscle, buffer solution and dye, shake vigorously and place in a thermostat at 37°.

(2) Prepare a second series of nine tubes as above but use the leuco-compound of Janus red instead of the dye itself. To prepare the leuco-compound, reduce a 0·02 per cent solution of Janus red with a small excess of hydrosulphite. Before adding the solution of the leuco-compound, evacuate the tubes thoroughly in order to remove the large amounts of oxygen always present in the washed muscle. Then add the solution of the leuco-compound (1 c.c. to each tube) and evacuate again.

(3) Prepare a third series of tubes as in (1) but use neutral-red solution (0·02 per cent) in place of Janus red.

(4) Make sure that in presence of sodium lactate the muscle pulp completely decolorises 0·02 per cent methylene-blue solution and 0·02 per cent indigo carmine solution. To do this use two groups of three tubes each, preparing them as in the case of the first three tubes of series (1).

Note the following points :

Series (1). Rapid decolorisation occurs in the tubes which contain Janus red and lactate, the process being almost complete after about 30 minutes. A very faint colour persists for a longer time.

In the tubes containing pyruvate, decolorisation is extremely slight.

In those tubes which contain equimolecular amounts of lactic acid and pyruvic acid about half of the colour disappears in the course of ½-hour. Here also a state of equilibrium is attained.

Series (2). With lactate alone no colour appears ; or at any rate, the faint colour which may be produced as a result of re-oxidation of the leuco-compound does not become deeper.

In the tubes with pyruvate alone, on the other hand, the dye is almost completely oxidised.

With the equimolecular mixture of lactate and pyruvate, oxidation of the leuco-compound occurs but ceases after 30 minutes when about half of the leuco-compound has been oxidised.

Series (3). Corresponding results are obtained with *neutral-red* but, on account of the more negative E_0-value of this dye, the equilibrium is attained in the equimolecular mixture of lactate and pyruvate when about 30 per cent decolorisation has occurred and the decolorisation with lactate is less complete than in the first series. Reduction and attainment of equilibrium take place more slowly.

Series (4). The samples with *methylene-blue* or *indigo carmine* and lactate are completely decolorised within a short time because the oxidation-reduction potentials of these two dyes are less negative than are those of the first two dyes used.

6. MANOMETRIC MEASUREMENT OF GAS METABOLISM BY WARBURG'S METHOD. OXYGEN RESPIRATION OF ACETIC BACTERIA

The manometric method worked out by Warburg is used for measuring the gas metabolism of cells. In this method the change in the pressure of the gas at constant volume and temperature is measured and, by means of the gas laws, the amount of gas which disappears or is produced is calculated. In biochemistry the method is chiefly used in studying :

1. **Animal and vegetable respiration** : consumption of oxygen ; a

series of intermediate products and finally carbon dioxide being produced.

2. **Assimilation of carbon dioxide** by green plants: consumption of carbon dioxide and production of oxygen.

3. **Fermentation processes** in which gas is produced or consumed. (*a*) *Alcoholic fermentation*: production of carbon dioxide. (*b*) *Oxidative fermentations*: acetic and propionic fermentations which involve consumption of oxygen.

4. **Fermentations** in which *lactic acid* is produced, this acid liberating from added hydrogen carbonate a corresponding amount of carbon dioxide: lactic acid fermentation, animal glycolysis.

Naturally, the method is suitable not only for experiments with living cell-material, such as yeast cells, bacterial cells, muscle pulp and sliced tissue, but, in the same way, for the study of materials which can be kept for longer periods, such as yeast and bacteria dried with acetone or cell-free enzyme preparations and solutions all of which take part in the metabolic processes mentioned: animal and vegetable oxidative enzymes, the zymase complex and so forth. The method is also perfectly suitable for use in *model experiments.*

Apparatus.—The original apparatus used by Barcroft consists of a respiration vessel and an attachment connected by means of a greased ground-glass joint to a capillary U-tube which contains the liquid used for measuring the pressure changes, namely, Brodie's solution[1] or mercury (10,000 mm. of Brodie's solution = 760 mm. of mercury). The attachment frequently has a cup inside and an opening which can be closed with a greased cap and the manometer is always fitted with a stop-cock which enables the internal pressure to be equalised with the external atmospheric pressure after the experiment is finished. During the experiment the vessels, other than the manometer itself, are immersed in water in a thermostat and shaken horizontally. In vessels on stands with graduated manometers (see Fig. 28) measurements at constant volume cannot be made; but the error produced in this way may be neglected. Warburg's apparatus which should be available for use in every large scientific institute gives more accurate results. In this apparatus the attachments (see Fig. 29) to the capillary manometer are suspended in the water in the thermostat. All the manometers are mounted on a movable frame fixed on the outer wall of the thermostat. Strips of squared paper pasted alongside the capillaries enable the pressure recorded to be read off. The capillaries are connected to rubber tubes also filled with Brodie's solution. This arrangement makes it possible to adjust the level of the liquid at any time to the zero point by turning a screw. A specially constructed shaking machine driven by an electric motor and having a speed control moves the frame with the manometers and attached vessels. The thermostat also contains an efficient stirrer and a thermo-regulator. Such an apparatus is shown in Fig. 30.

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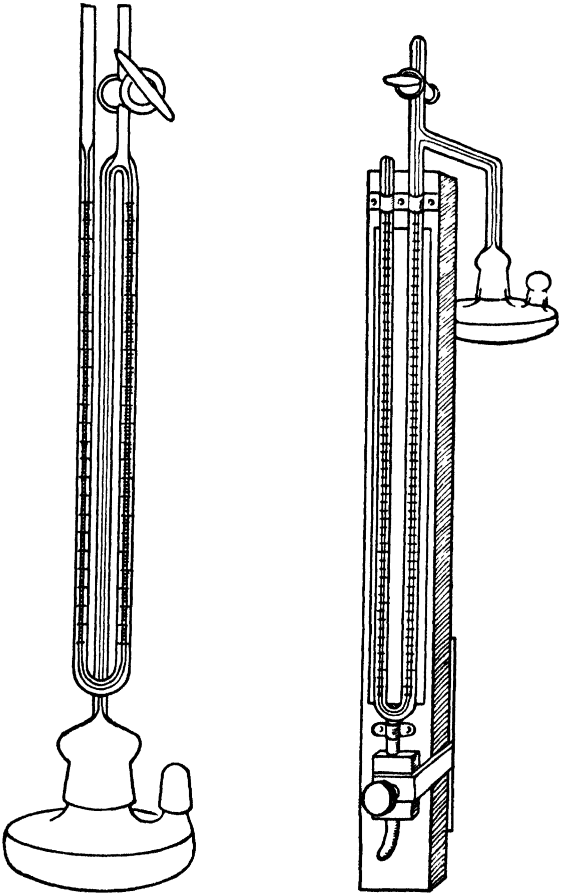
[1] Preparation of Brodie's solution: dissolve 23 g. of common salt and 5 g. of the sodium salt of choleic acid in 500 c.c. of water and add a few drops of concentrated alcoholic solution of thymol. Colour the solution with eosin.



FIGS. 28 and 29.

Calibration of the vessels. In order that the readings of the manometer (mm. of Brodie's solution or mercury) can be related to the volumes of gas (c.c.) the capacities of the vessels must be known. These are most accurately determined by weighing the vessels filled with mercury or water. This procedure is particularly advantageous in the case of vessels on stands. The mercury is poured in to the level of the zero mark. The manometric method of calibration is simpler but cannot be used for vessels on stands. Proceed as follows: suspend the *moist* respiration vessel in the thermostat at room temperature. Open the stop-cock and lower the measuring liquid by an amount h (10–20 mm.) below the zero point. Close the cock and, by turning the screw, adjust the level of the liquid in the right-hand leg of the U-tube to zero. The level in the left-hand leg is thus raised by the amount h_1. Repeat the process after pouring a c.c. of liquid into the respiration vessel ($a = 5$–20 c.c. of water). This time the liquid in the left leg rises higher—by the amount h_2. On both occasions the amount x of gas is compressed in this way in the respiration vessel. According to Boyle's law:

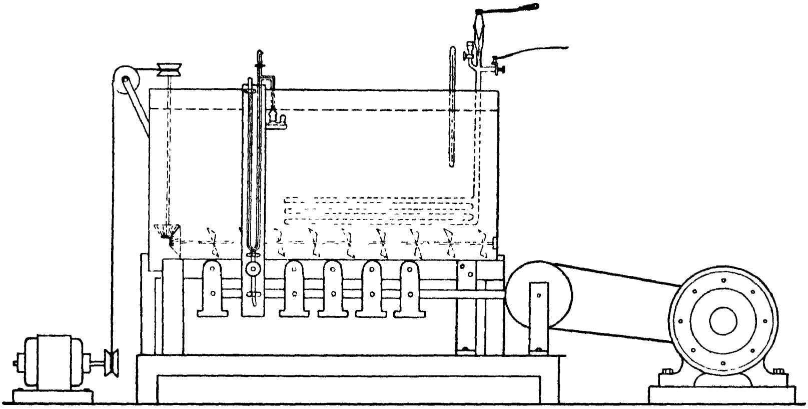


FIG. 30.

I. $(P+h_1)v=P(v+x)$

II. $(P+h_2)(v-a)=P(v-a+x)$

Whence it follows that:

$$\text{Volume } v = a\,\frac{h_2}{h_2 - h_1}$$

P is the atmospheric pressure.

The "constants" of the respiration vessels, which are obtained by determination of their capacities, are used in *calculating* the gas exchange. These constants are calculated by means of Warburg's formula :

$$K = \frac{v_G \frac{273}{T} + v_F \alpha}{P_0}, \quad . \quad . \quad . \quad (A)$$

where v_G is the volume of gas in the respiration vessel ;
v_F ,, ,, liquid ,, ,,
T ,, absolute temperature ;
P_0 ,, normal pressure (760 mm. of mercury or 10,000 mm. of Brodie's solution) ;
a ,, absorption coefficient of the gas which is produced, or is present and consumed.

The volume V of the gas, which is to be determined, is calculated by means of the formula $Kh = V$, where h is the manometer reading in mm. of mercury or Brodie's solution. According to the nature of the gas, having partial pressure h, K, the constant of the respiration vessel, varies. Values of a are given in Landolt-Börnsteins *Tabellen.* Thus there is a K_{O_2}, a K_{CO_2} and so on. Naturally, the value of the constant depends also on the amount of liquid in the respiration vessel.

Formula (A) always holds in such cases in which the partial pressure of *one* gas only changes, namely, in oxidative fermentations, anaerobic fermentation with yeast, anaerobic lactic acid fermentation, in presence of hydrogen carbonate, and so forth. It may also be applied in such cases of the study of gas metabolism where normally the partial pressures of *two* gases would change, were it not that, as a result of the presence of absorbents (potassium hydroxide for CO_2, palladium on asbestos for hydrogen), the change in the partial pressure of only *one* gas is measured by means of the manometric reading. Examples are : respiration processes in the presence of potassium hydroxide, bacterial fermentations in which there occurs simultaneous liberation of carbon dioxide and hydrogen. When *several* gases simultaneously disappear or are produced as, say, in the process of respiration and aerobic fermentation with yeast where oxygen is consumed and carbon dioxide is produced, other formulae, also worked out by Warburg,[1] hold for calculating the constants of the vessels. Attention may be drawn to an experimental procedure developed by Warburg which is also applicable in these cases and involves the use of simple constants.[1] When phosphates of the type X_2HPO_4 (X = univalent metal) are used, the "retained" carbon dioxide must be determined.[1, 2]

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[1] See Oppenheimer, *Methodik der Fermente,* vol. iii., p. 648.

Experiment.—Study the oxidation of lower alcohols and of acetaldehyde by acetic bacteria (dehydrogenase of acetic bacteria) by manometric measurement of oxygen uptake in six Barcroft vessels.[3] Carry out this experiment as a *parallel* to those described on pages 160 and 166 making up the samples to correspond as far as possible with those used in the earlier experiments. Use as donators: (1) methyl alcohol; (2) ethyl alcohol; (3) *iso*propyl alcohol; (4) acetaldehyde; (5) ethyl alcohol in presence of 0·002*N*-HCN. Put no donator in the sixth vessel but use it for determining the residual respiration. Titrate the *acid* produced by dehydrogenation of the primary alcohols and acetaldehyde with 0·05*N*-NaOH solution, using phenolphthalein as indicator, and note the relation between the amounts of acid and those of oxygen consumed. If desired, take aliquot portions of the samples obtained from the primary alcohols and show that aldehyde is produced.

Vessel No. . .	1	2	3	4	5	6
Suspension of bacteria (5 mg. dry matter per c.c.) . . .	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
0·2*M*-Acetate buffer solution, p_H 5·6 . .	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
0·1*M*-Donator (1 c.c. of each) . . .	Methyl Alcohol	Ethyl Alcohol	*iso*Propyl Alcohol	Acetaldehyde	Ethyl Alcohol	—
0·02*M*-Hydrocyanic acid solution . . .	—	—	—	—	1 c.c.	—
Water (c.c.) . . .	7	7	7	7	6	8

Procedure.—Prepare six Barcroft vessels of known capacity with material as shown in the table, connect to the manometers (grease carefully!), make fast with springs or rubber bands, open the stopcocks and in order to produce temperature equilibrium shake for 10 minutes at a temperature (28°–30°) which is kept constant throughout the experiment. Pour 10 c.c. of water into each of two additional vessels which thus contain the same volume of liquid as do the other vessels and place in the thermostat with the others.

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[1] *Biochem. Z.*, 1924, 146, 380.

[2] See Oppenheimer, *Methodik der Fermente*, vol. iii., p. 648.

[3] Bertho, *Annalen*, 1929, 474, 21.

These two serve for checking the temperature. Adjust the surface of the liquid columns in the manometers to the zero mark which is preferably in the middle of the scale. After temperature equilibrium has been reached, rapidly close the cocks without stopping the shaking and take periodical (5–30 minutes) readings on the manometer scales according to the requirements of the experiment and the magnitude of the movements of the liquid in the manometers. Take the reading by turning the screw so that the surface of the column in the leg directly connected with the vessel is depressed until it reaches the zero mark (when gas is evolved raise the column). Then read the level on the other leg noting the reading and the time—preferably in a small table. After 90–120 minutes take out the vessels and titrate the whole contents—if desired, after removing 1 c.c. from each vessel for testing for aldehyde—of vessels 1, 2, 4, 5 and 6 with 0·05 N-sodium hydroxide solution from a micro-burette, using phenophthalein as indicator. In order to determine the actual amount of alkali consumed by the acid produced carry out a *blank experiment* in duplicate before beginning the experiment and deduct the value found from the results obtained afterwards. If it is assumed that the oxygen consumed in the dehydrogenation of donators is converted into water, then according to what was said about acetic fermentation on p. 165 $\frac{1}{2}O_2$ is required to dehydrogenate alcohol to the aldehyde stage and likewise $\frac{1}{2}O_2$ to convert the aldehyde into the acid and hence to convert the primary alcohol into the acid O_2 is required. Calculate the number of cubic centimetres of oxygen consumed in each vessel taking into account the constants of the vessels and relate to the amounts of acid produced. 2 c.c. of 0·05 N-sodium hydroxide solution are equivalent to the same volume of acid of the same normality and to 2240 c.c. of oxygen (conversion of alcohol into acid); or to 1120 c.c. (conversion of aldehyde into acid). For accurate calculation take account also of the oxygen consumed during the 10 minutes occupied for attaining temperature equilibrium. It will be found that for vessels 1 and 2 the titration value obtained is too small when related to the oxygen consumption. This is so because, as in the acceptor experiments, the alcohol is only partly oxidised to the aldehyde stage. In experiment 4 (acetaldehyde) the values agree well. A small excess of acid (5–10 per cent) is produced by *dismutation*. In vessel 5 where the aerobic biological oxidation is strongly inhibited by the high concentration of hydrocyanic acid there is no appreciable consumption of oxygen and the amount of acid produced is inconsiderable. So also in vessel 6 since the residual respiration is very slight.

Compare the decolorisation times for methylene-blue observed in the experiment described on p. 160 and the amounts of quinone transformed in that described on p. 166 with volumes of oxygen consumed. It will be found that some degree of parallelism exists between these volumes, the amounts of quinone transformed and the reciprocals of the decolorisation times. Exceptions to this are the results of the experiments with hydrocyanic acid since this acid has no notable effect on respiration with acceptors whilst it strongly inhibits respiration with oxygen. This fact can be very elegantly demonstrated as follows: add to a sample poisoned with hydrocyanic acid 1 c.c. (0·5 mg.) of methylene-blue solution. A very considerable increase in the oxygen uptake results because the dye acts as an acceptor being converted into leucomethylene-blue which is immediately auto-oxidised with consumption of oxygen. Thus methylene-blue acts as carrier and catalyst (see p. 226).

On the relations between the transformations with the three acceptors, see p. 195.

The *oxidation of alcohol* to acetaldehyde and acetic acid by means of *acetic bacteria* as in the experiments described proceeds under conditions different from those obtaining in normal *acetic fermentation.* In the experiments, samples were studied under definite conditions (temperature, pressure, concentration) in order that the dehydrogenase of the acetic bacteria could be investigated according to the principles of chemical technique. In acetic fermentation, on the other hand, the normal substrates are poor quality wine, mashes containing alcohol and so forth. Here growth processes are not excluded.

According to Wieland's view, as was pointed out in detail on p. 165, acetic acid production takes place as follows: hydrogen activated by a specific enzyme (dehydrogenase) is transferred, two atoms at a time, from the alcohol or aldehyde hydrate (donators) to molecular oxygen. Thus, *hydrogen peroxide* must be produced in the first instance. This substance is a poison for the cells and it is decomposed into water and oxygen by the *catalase* which abounds in acetic bacteria (see p. 201). Catalase is sensitive to the action of hydrocyanic acid. Acetaldehyde as intermediate product is transformed chiefly by dehydrogenation with oxygen. Acetic bacteria contain, in addition, a complex enzyme system (*mutase* and *co-mutase*) which can dismute the aldehyde, as in a Cannizzaro reaction, into acetic acid and alcohol. Warburg does not deny the existence of dehydrogenating systems but believes that the chief part is played by oxygen activated by enzymes containing iron (respiratory enzymes). He bases his argument mainly on the sensitivity of biological oxidations towards hydrocyanic acid. According to Wieland this acid acts by inhibiting the action of the catalase which should destroy the hydrogen peroxide. Warburg's views are considered at length on p. 222.

THE DEHYDROGENASES. A SUMMARY

The dehydrogenases are the most labile bio-catalysts known. It has indeed been possible, in various ways, to separate some dehydrogenases from the cells to which they are bound, and to study them in solution, but the low degree of stability of the solutions and the loss of activity which results from the dissolution have unfavourable effects on the investigation. The Schardinger enzyme of milk, on the other hand, occurs in the cell-free condition, is relatively stable and can also be obtained in the form of dry material (see p. 169). The methods of enzyme chemistry either are inadequate for the study of the dehydrogenases—these methods were developed for the hydrolytic enzymes—or else, more probably, many dehydrogenases are united to the structural elements of the cells and therefore cannot be separated from them at all. The *dehydrogenases of the acetic bacteria*, for example, cannot be obtained in the cell-free condition. Accordingly, in many cases, the study of dehydrogenases must be confined to their compounds with the cells. For this purpose, it is preferable to use unicellular organisms, such as bacteria or yeast or else finely divided cell-tissue such as muscle pulp. Living cells are used and also cells in which the vital functions have been largely or completely destroyed by treatment with toluene or acetone[1] or by drying.

When *living unicellular organisms* are used, that is to say, macro-heterogeneous systems the question arises whether the results obtained with such *dehydrogenase-containing material* may be applied in the same way as are the results obtained with dissolved dehydrogenases. The transformations in the dehydrogenase systems may be decisively altered by *adsorption* at the surface of the cell and by the *permeability* of the cell membrane. When *hydrogen donators not foreign to the cells*, such as glucose, alcohol, acetaldehyde, are used no such difficulties arise. This behaviour is in harmony with Willstätter's observation that the action of invertase is independent of its degree of freedom, that is to say, of whether it is bound to the cell, adsorbed or free. The *biological acceptors* also, above all oxygen, are no exceptions to this rule. All experience shows that quinone also, despite its low degree of surface-activity, has free access to the points where the dehydrogenases are located : only the tanning effect interferes to some extent.

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[1] See, for example, Buchner and Gaunt, *Annalen.*, 1906, 349, 140.

When *dyes* (methylene-blue for example) are used as *acceptors*, however, the difficulties mentioned are encountered. When the transformations produced by methylene-blue, quinone and oxygen in the experiments described on pages 160, 167 and 191 are compared in terms of unit of time and constant amount of *bacteria* the ratio in the most favourable case is 1 : 30 : 12. The low activity of methylene-blue as acceptor is chiefly due to the low permeability of the outer layers of the cells to the dye : the rate of diffusion of the methylene-blue into the cell determines the extent of the dehydrogenation. In *yeast* and *muscle* also the transformation with oxygen is very much greater than with methylene-blue. But there are cells with which these differences in the action of acceptors do not arise, especially cells, such as those of *lactic acid bacteria*[1] for example, which are free from haemin or related compounds. Hence the reasons for the differences are not quite clear and some support is provided for Warburg's assumption that the inferiority of methylene-blue as acceptor is to be traced to the action of cells which contain haemin. He supposes that methylene-blue does not react directly with the activated hydrogen of the substrate but must make use of the haemin of the cells as an intermediate (see p. 225).

The *dehydrogenases* exhibit pronounced *specificity towards donators*. Many of them, such as *succino-dehydrogenase* for example, exhibit perfectly clear specificity towards a single donator (see p. 152). With other dehydrogenases the zone of specificity extends to several donators having constitutional or configurative relationships with each other. Such is the *dehydrogenase of the acetic bacteria* for example. Dehydrogenases of this type are *group-specific*. When several donators are dehydrogenated by one and the same dehydrogenase the amount of acceptor consumed in a sample containing the dehydrogenase and two or more of these donators in optimal concentration should exhibit no increase. Hence this absence of *additive effect* is an indication that one and the same dehydrogenase reacts with various donators.

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[1] Bertho and Glück, *Annalen*, 1932, **494**, 159.

The original conception that each thermodynamically possible hydrogen acceptor must be able to take up the hydrogen activated by the enzyme cannot be retained. It is true that the acceptor must have a higher oxidation-reduction potential than has the system undergoing dehydrogenation but this is not a sufficient condition for the occurrence of dehydrogenation. The fact that in many cases acceptors, which fulfil the thermodynamic conditions, do not react at all and also the results of certain kinetic investigations, which cannot be considered in detail here, have led to the supposition that the dehydrogenases also exhibit *specificity towards acceptors* (Bertho [1]). Thus the dehydrogenases exhibit *enzymic specific affinity* both to donator and acceptor. This harmonises completely with the strict specificity which biological catalysts exhibit towards the substances available to the cells. The existence of aerobic and anaerobic dehydrogenases illustrates this clearly.

According to the modern conception of the chemistry of enzymes the action of these is due actually to *addition compounds of enzyme and substrate* (see p. 128). The hydrolytic enzyme because of its specific affinity reacts only with one kind of molecule to form an enzyme-substrate compound. In the case of the enzymes which transfer hydrogen, namely, as has been mentioned, the enzymes which exhibit double specificity, study of the kinetics of the reaction shows that a *ternary complex of donator-enzyme-acceptor* is to be held responsible for the enzymic action (Bertho [1]). The activated and mobilised hydrogen atoms of the donator which is absorbed on the enzyme are transferred to the acceptor which is also adsorbed on the enzyme. When the ternary complex decomposes the free enzyme, the dehydrogenation product and the hydrogenated acceptor are produced. These views were first developed in the study of the dehydrogenase of the acetic bacteria (Bertho) and have been shown to be capable of application to the Schardinger enzyme (Wieland). The original literature must be consulted for further details.

One point in connexion with the action of *oxygen* as acceptor, that is to say, in the *respiratory process*, is especially striking. It is

[1] *Annalen*, 1929, 474, 1.

observed almost throughout that the velocity of reaction, until the concentration (pressure) of oxygen has become very low, is independent of the oxygen concentration (pressure) whilst with the other acceptors a distinct dependence of the velocity of reaction on the concentration of the acceptor is observed. If, in accordance with the views expressed above, oxygen like any other acceptor is adsorbed on the active surface of the dehydrogenase, then it is obviously permissible to speak of " activation of oxygen on the dehydrogenase " (see p. 229). The absence of dependence on concentration mentioned above is caused then by the extraordinarily *high affinity of oxygen for the dehydrogenase.* On the other hand, the special position of oxygen (see p. 222) can likewise be explained by assuming the existence of a special enzyme which activates oxygen (Warburg.)

The action of the dehydrogenases is either not inhibited at all by *hydrocyanic acid* and *carbon monoxide,* or at least is much less inhibited than is that of the haemin-containing oxidative enzymes described in the following section.

II. OXIDATIVE ENZYMES CONTAINING HEAVY METAL

Chemistry of Haemin

The dehydrogenation theory is in no way concerned with the possible presence of heavy metals in the dehydrogenating enzymes or indeed with their chemical nature at all. Very recently, however, studies have been made of the chemical nature of certain oxidative enzymes. These studies have been initiated chiefly as a result of Warburg's hypothesis that the *respiration of cells is a catalytic process* where the catalyst is a *heavy metal* and that *iron, in the form of a widely distributed haemin-like compound* the so-called *respiratory enzyme,* may be the activator of oxygen. When haemin complexes were sought in other isolated and highly purified oxidative enzymes the search was successful and the complexes were actually found in *catalase* and the *peroxidases.* Finally, the *haemins of the cells* and particularly the so-called *cytochrome,* play, according to Keilin's view, a very special part in the respiratory process. This view is a compromise which includes at the same time views about the dehydrogenating and oxygen-activating processes and enzymes.

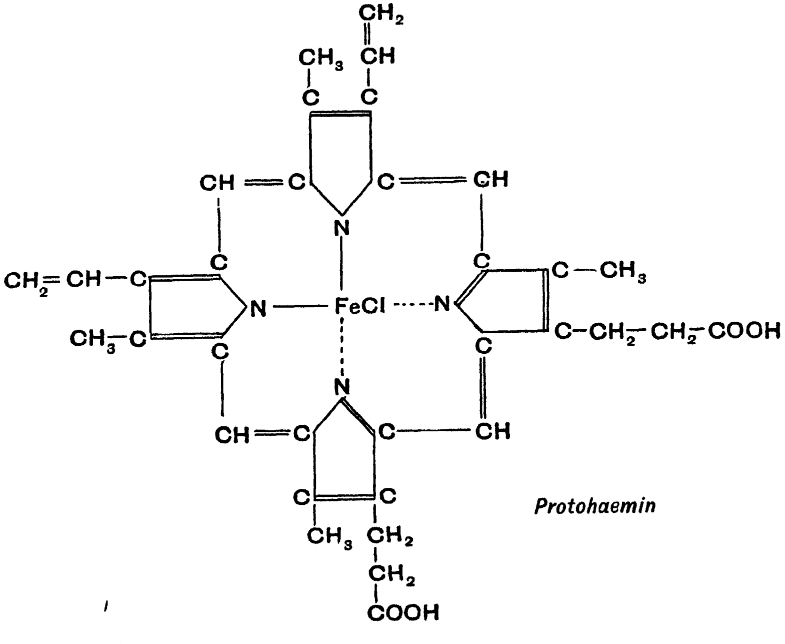
Indeed, attempts to form such hypotheses are receiving more and more support in all branches of investigation.

The *haemoglobin* of blood has no enzymic properties. It forms a loose compound with oxygen, namely, oxyhaemoglobin, and serves as a carrier of oxygen which it transports to the points where oxidation occurs.

The discovery that haemin occurs in at least four enzymes made it necessary to find out whether, in addition to this haemin, there occurred in the cells free haemin or haemin in non-specific combination apart from the haemin of the blood. It may be assumed that this is certainly so. But the functions of the so-called *free haematin* in the process of oxidation have not yet been made clear.

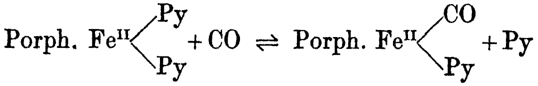
The most important points in the chemistry of haemin must first be discussed. The red pigment of the blood, haemoglobin, consists of two components, the *prosthetic group* of the molecule, that is to say, the pigment and the colourless protein, namely, *globin.* Glacial acetic acid in the presence of sodium chloride removes the globin and a crystalline substance, in the form of the so-called Teichmann crystals, is obtained. These are crystals of haemin (also called chlorohaemin) which has the composition $C_{34}H_{32}O_4N_4FeCl$ (see Gattermann: *Laboratory Methods of Organic Chemistry*, p. 407, London, 1937). The iron can easily be removed from this compound leaving *porphyrin,* a substance containing four pyrrole rings. Porphyrins of this type whether free from or containing metal are widely distributed in the animal and vegetable organisms. The porphyrin which is derived from haemoglobin in *protoporphyrin* and the corresponding haemin is *protohaemin.* Protoporphyrin is obtained from the haemin of blood by the action for formic acid and iron, which removes the tervalent iron from the pigment. Protoporphyrin is also obtained directly from haemoglobin by the action of hydrochloric acid. The formula shown below is that of H. Fischer and K. Zeile who have confirmed their view by synthesis. Results so far obtained show that protohaemin is identical with or extraordinarily closely related to the haemin components of peroxidase, catalase and cytochrome-*C*. It seems that the haemin of the respiratory enzyme is of a special type (see p. 224).

Appropriate text-books must be consulted for details of the properties and formation of the natural and synthetic porphyrins. Here only those types of haemin which play a part in the oxidation processes in cells will receive attention. When the chlorine of *chlorohaemin* (Fe^{III}) is replaced by hydroxyl, *haematin* (Fe^{III}) is obtained. Hydrosulphite, cysteine and so on, convert haematin into *haem* (Fe^{II}) which is extraordinarily easily oxidised by molecular oxygen. Neutral haematin combines with denatured globin, denatured proteins and bases to give the so-called *parahaematins* (Fe^{III}). In an exactly similar way reduced haematins or haems combine in alkaline or neutral solution with denatured proteins and various



Protohaemin

bases, such as pyridine, nicotine, caffeine, ammonia and so forth, as well as with cyanide to give *haemochromogens* (Fe^{II}) which exhibit especially characteristic absorption spectra. In the haemochromogens 1 molecule of haem is combined with 2 molecules of the nitrogenous component. The haemin derivatives containing *bivalent* iron, namely, haem, haemochromogen and haemoglobin, combine with *carbon monoxide* in contrast to those with *tervalent* iron haematin, parahaematin and methaemoglobin, whilst these three like the first three, however, are able to combine with *hydrocyanic acid.* The compounds produced exhibit pronounced light absorption. These facts are of interest when it is recalled that peroxidase and catalase, which are believed to contain tervalent iron, are not sensitive to the action of carbon monoxide but are sensitive to that of hydrocyanic acid. Haem combines with 1 molecule of carbon monoxide; in haemochromogen 1 molecule of the base is expelled by 1 molecule of carbon monoxide thus:



$$\text{Porph. Fe}^{\text{II}}\begin{array}{l}\diagup\text{Py}\\ \diagdown\text{Py}\end{array} + \text{CO} \rightleftharpoons \text{Porph. Fe}^{\text{II}}\begin{array}{l}\diagup\text{CO}\\ \diagdown\text{Py}\end{array} + \text{Py}$$

(Py = pyridine)

Carbon monoxide-haemochromogen is sensitive to the action of light. In the presence of excess of pyridine the above equilibrium is shifted from right to left on irradiation.

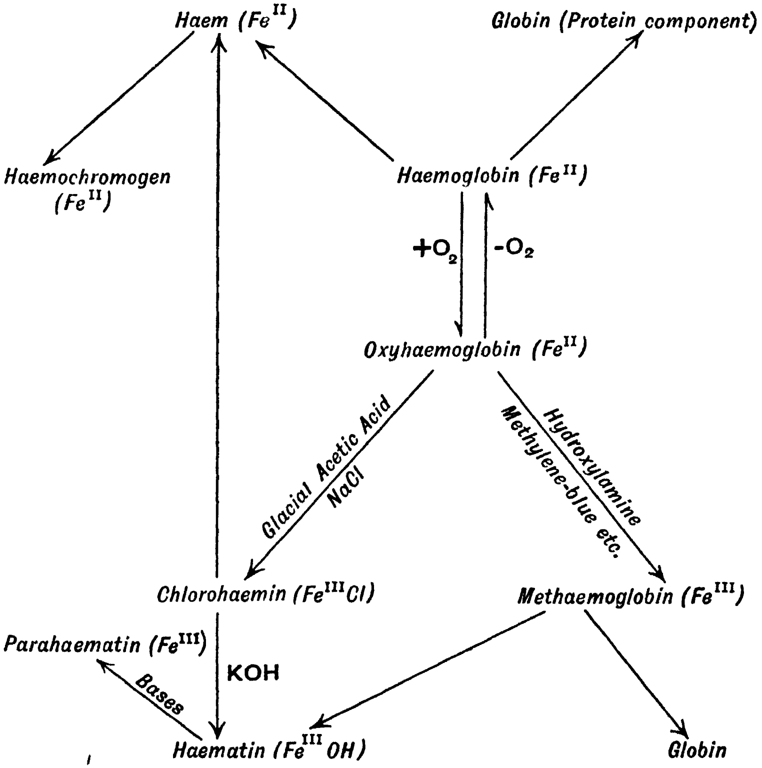
The conversion of haemoglobin (Fe^{II}) into *oxyhaemoglobin* is not accompanied by a change in the valency of the iron. The process which occurs here may be called *oxygenation* for the oxygen in oxyhaemoglobin is only loosely combined. In order to convert the iron of haemoglobin or oxyhaemoglobin into *ter*valent iron [production of methaemoglobin (Fe^{III})] mild oxidising agents, such as hydroxylamine, methylene-blue, potassium ferricyanide, must be used whilst the two other ferrous compounds, haem and haemochromogen, are auto-oxidisable to haematin and parahaematin respectively. Conversely, the ferri-compounds can be reduced.

In all animal haemoglobins the *prosthetic group* is the same, namely, *reduced protohaematin*. Differences between haemoglobins with regard to adsorption, behaviour towards CO and so on, must be traced exclusively to the protein component, namely, globin. In the artificial haemochromogens, however, the structure of which corresponds to that of the natural haemoglobins, the absorption spectrum is affected by the nature of the nitrogen compound of the haemin portion and by the degree of dispersion of the compound. These facts must be borne in mind when the haemins of cells are studied spectroscopically (see cytochrome).

The scheme below shows the interrelationship between the compounds. It is specially indicated, in the case of the iron compounds, whether the element is in the ferrous or ferric condition.

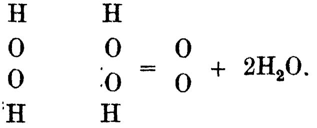
Haemoglobin, *oxyhaemoglobin*, *haem* (the very easily auto-oxidised constituent of haemoglobin) and the *haemochromogens*, and the double compounds of haem with bases (such as pyridine, nicotine and so on) which can be isolated and which have the general formula $\text{Fe}^{\text{Py}}_{\text{Py}}$ (Py = pyridine) contain, accordingly, *bivalent iron*;

methaemoglobin, *chlorohaemin*, *haematin* and *parahaematins*, the double compounds of haematin and nitrogen compounds, on the other hand, contain *tervalent* iron.



1. Catalase

In all aerobic organisms there occurs an enzyme, namely, catalase, the specific function of which is to decompose *hydrogen peroxide*. It has no other known specific action. Attempts have been made to ascribe to catalase a part in the *respiratory process*. Anaerobic organisms contain no catalase. But up to the present it has not been possible to show with absolute certainty that the rôle of catalase is significant and necessary for the respiration of cells. No relation exists between oxygen consumption and catalase content. The dehydrogenation theory provides a general explanation of the functional significance of catalase in oxidations in the cells. Since the first product to be expected in dehydrogenation by molecular oxygen is *hydrogen peroxide*, and since this compound, being a powerful poison for the cells, would be detrimental to the organism, the function of catalase is to destroy this poison (see p. 150). According to Wieland, the reaction takes the form of an intermolecular dehydrogenation, thus :



$$\begin{matrix} H & H & & \\ O & O & & O \\ O & O & = & O \\ H & H & & \end{matrix} + 2H_2O.$$

In many cases catalase acts as a protective enzyme. When hypoxanthine is aerobically oxidised by xanthine dehydrogenase, hydrogen peroxide (see p. 172), which damages or destroys the dehydrogenase, is produced. In the presence of catalase, however, the dehydrogenation continues. The production of oxygen from the hydrogen peroxide by the catalase satisfies the requirements of the cell.

Like the respiratory process, catalase is sensitive to the action of *hydrocyanic acid*—another indication that the enzyme plays a part in respiration. This sensitivity it shares also with colloidal platinum, an inorganic catalyst which closely resembles catalase in its effects and which also decomposes hydrogen peroxide (see p. 146).

Catalase is very widely distributed in nature and is especially abundant in mammalian liver and blood. Mammalian liver, especially horse liver, is the most convenient starting material for use in preparing the enzyme. The most active preparations are those obtained by the methods of Hennichs, von Euler, Josephson and Zeile. A description follows of a method of purification involving precipitation with alcohol, adsorption and elution.

Preparation of Catalase.[1]—Mince finely 0·2 kg. of horse liver and extract with 200 c.c. of water by shaking for 1 hour in a machine. Cool the extract, add a half volume (100 c.c.) of alcohol and centrifuge in order to remove the precipitated protein. Then add the same volume (100 c.c.) of alcohol and, in order to remove haemoglobin and residual protein, 100 c.c. of chloroform and shake for a short time. Now centrifuge the emulsion produced. The denatured haemoglobin separates at the liquid interface in the form of a solid cake. The yellowish solution (volume about 300 c.c.) contains all the catalase, is free from haemoglobin and has a " catalase value " of about 4000–5000.

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[1] Von Euler and Josephson, *Annalen*, 1927, 452, 174; Zeile and Hellström, *Z. physiol. Chem.*, 1930, 192, 172. For the preparation of crystalline catalase see Sumner and Dounce, *Science*, 1937, 85, 366; *J. Biol. Chem.*, 1937, 121, 417.

For further purification adsorb the enzyme on tricalcium phosphate[1] and then elute with 1 per cent solution of disodium hydrogen phosphate. For the preparation of the tricalcium phosphate see below.

Stir the solution of the enzyme well with a suspension of the tricalcium phosphate (3 g. in 100 c.c., *i.e.* a 3 per cent suspension), centrifuge and elute with 75 c.c. of 1 per cent solution of disodium hydrogen phosphate. The solution obtained is twice as active as that used for the adsorption : use it alone for the dialysis which follows. (Second and third elutions each with 75 c.c. of liquid yield less concentrated extracts. In all, 70 per cent of the catalase is obtained by elution.)

Dialyse at low temperature the solution (volume about 70 c.c.) obtained at the first elution using a parchment membrane.[2] Prevent " creeping " of the solution by greasing the rim of the thimble with some lanoline. About 30 per cent of the catalase is lost during the dialysis, but even a single dialysis results in considerable purification. The catalase value of the solution (best kept under toluene in the refrigerator), which is measured in the manner described below, amounts to 30,000 or somewhat less. Determine the amount of dry material in an aliquot portion of the solution so that the determination of activity may be possible. The solution has an olive-green colour and exhibits a red fluorescence produced by the haemin component of the catalase.

The solution is extraordinarily active. In order to prevent considerable loss of activity never dilute with water but always with buffer solution. Water may be added if desired to the diluted solution.

Preparation of Tricalcium Phosphate.—Dissolve 7·6 g. of trisodium phosphate $Na_3PO_4.12H_2O$ and 6·57 g. of calcium chloride $CaCl_2.6H_2O$ each in 100 c.c. of distilled water and mix the two solutions. In order to remove sodium chloride from the precipitate produced, add to it distilled water and stir well. Let the solid then settle and draw off the supernatant liquid. Repeat the stirring with water until this liquid gives no turbidity with nitric acid and silver nitrate. Then separate the precipitate from the liquid by centrifuging once and suspend in 150 c.c. of water. Keep the suspension thus prepared in a stoppered flask in the refrigerator.

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[1] Tsuchihashi, *Biochem. Z.*, 1923, 140, 63.

[2] Still better is a cellophane membrane.

Methods of Determination.—The catalase content of a preparation is determined by measuring the amount of H_2O_2 which it decomposes. Either the amount of oxygen liberated is determined volumetrically or the unchanged hydrogen peroxide is titrated with *permanganate* or *iodine* solution. It is convenient to use as unit the *catalase value* which is determined at a defined temperature, usually 0°, and at the optimal p_H for catalase, namely, 6·8–7·0 ($M/150$ phosphate buffer solution). The concentration of hydrogen peroxide should amount to 0·01–0·02 mole.

$$\text{Catalase value} = \frac{\text{Reaction constant } k \text{ at } p_H \text{ 6·8–7·0 in 0·015 mol. } H_2O_2}{\text{g. of enzyme preparation in 50 c.c.}}$$

When the hydrogen peroxide concentration is not altogether too high, say not more than $0·02M$, the reaction is of the first order and k is then the reaction constant for a unimolecular reaction. The constant is determined by the equation :

$$10^4 k = \frac{4343}{t} \cdot ln \frac{a}{a-x},$$

where a is a determined concentration, preferably the initial concentration and $a-x$ is the concentration after the lapse of time t. The figure 4343 serves to convert natural into common (Brigg's) logarithms. The results of the two methods do not agree well. Since, in the first method, the collection of the oxygen in the eudiometer requires time, the results are always low. The titration method is usually to be preferred.

Within the limits of hydrogen peroxide concentration given, the reaction, as has already been said, is of the first order. Deviations from this order, such as those which occur at high hydrogen peroxide concentrations, are always attributed to destruction of the enzyme.

Virtanen uses the expression :

$$\text{Catalase value} = \frac{k}{\text{No. of cells}},$$

for the catalase of bacteria basing his view on the formula of Euler, namely :

$$\text{Catalase value} = \frac{\text{Reaction constant } k}{\text{No. of g. of enzyme in 50 c.c.}}.$$

The number of cells must be determined by means of a microscopic count, using a definite amount of cells.

Experiment.—To determine the catalase value of the enzyme material prepared from horse liver, using 0·015*M*-hydrogen peroxide solution.

(1) By measurement of the oxygen liberated.

(2*a*) By titrating the undecomposed hydrogen peroxide with permanganate after various intervals.

(2*b*) By proceeding as in 2*a* but after poisoning with 0·001*M*-hydrocyanic acid solution.

Procedure.—The following solutions are required :

1. 0·15*M*-hydrogen peroxide solution obtained by diluting Merck's perhydrol.
2. 0·1 *N*-$KMnO_4$.
3. 0·1*M*-HCN.
4. 0·067*M*-phosphate buffer solution (p_H 7·0).
5. A catalase solution containing a known amount of the enzyme (dry matter).

It is advisable to discover in all cases by means of preliminary experiments the approximate catalase content in order that the periods of reaction may be neither too long nor too short. In (1) and (2*a*) the most suitable periods are 30 to 60 minutes. If the catalase preparation available should not go completely into solution no harm is done : a small amount of flocculent material does not interfere. It is better, however, to use highly purified solutions containing known amounts of dry matter. As regards the dilution of such solutions see p. 203.

(1) **Volumetric Method** (*measurement of volume of gas*).—Pour into a thick-walled flask (capacity about 100 c.c.) 5 c.c. of 0·067*M*-phosphate buffer solution of p_H 7·0, 5 c.c. of 0·15*M*-H_2O_2 solution and 38 c.c. of water. Cautiously bring into the flask a short test-tube containing 2 c.c. of catalase solution and a piece of stout glass rod about 2 cm. long, taking care that the solution of the enzyme does not come into contact with the substrate. (A glass rod may be used to assist in the insertion of the tube.) Leave the flask unstoppered in ice-water for some time, then insert a rubber stopper carrying a gas delivery tube attached to a eudiometer almost filled with saturated sodium chloride solution. After some time read the level of the solution in the eudiometer in the usual way. Now shake the flask vigorously so that the test-tube containing the

enzyme is shattered and the reaction initiated. Note the time (*start of the experiment*). Measure the volume of gas in the eudiometer at suitable intervals (say, every 2–4 minutes) noting the time on each occasion. Throughout the experiment keep the flask in ice-water and shake mechanically or with the hand in order that regular evolution of gas may occur. The final volume of gas corresponds to 100 per cent transformation.

(2) **Titration Method.**—Proceed as in (1) but take double volumes of the liquids. Place in ice-water and wait until the mixture (*a*) has attained constant temperature before breaking the tube containing the enzyme. With a second sample (*b*) proceed in the same way but instead of taking 76 c.c. of water, take 75 c.c. and add 1 c.c. of $0{\cdot}1M$-hydrocyanic acid. Then add the enzyme solution—in each case 4 c.c. Note the time at which this is done (*start of the reaction*). Directly after doing so, remove an aliquot portion of sample (*a*) (10 c.c.) with a pipette, and run into a conical flask containing about 30 c.c. of dilute sulphuric acid. (This prevents the enzyme from acting.) Titrate with $0{\cdot}1\,N$-permanganate. Still better, titrate separately one-tenth of the amount of hydrogen peroxide taken. Repeat the titration at suitable intervals—every 2–4 minutes in the sample without hydrocyanic acid and every 5–10 minutes in that with hydrocyanic acid until the value found no longer alters. On each occasion note the time and the volume of permanganate used.

1. Compare the percentage transformations at the same times in (1), (2*a*) and (2*b*).
2. Determine the reaction constants ($k \times 10^4$) for (1), (2*a*) and (2*b*). k_1 and k_{2a} should agree theoretically. But for the reason given on p. 204 k_{2a} is always the larger. k_{2b} is considerably smaller.
3. Plot all the results on logarithmic paper—abscissae, time in minutes; ordinates (in logarithmic measure) c.c. of $0{\cdot}01\,N$-H_2O_2 consumed. The curves obtained should be straight lines since a logarithmic function is being plotted.
4. Calculate the catalase values for (1), (2*a*) and (2*b*) on the basis of grammes of enzyme per 50 c.c.

Catalase was the first oxidative enzyme in the highly purified solutions of which a *haemin complex* was directly detected by means of the spectroscope (Zeile and Hellström [1]). The materials used for the detection were highly purified products having a catalase value of 40,000. In these products the ratio of activity to haemin content was constant. It follows that haemin is the *active constituent.* It is very probable that the haemin of catalase is identical with that of blood (protohaemin). Little is known about the colloidal carrier. The catalase from the seeds of the pumpkin exhibits a spectrum identical with that of the catalase of horse liver. Solutions of catalase from horse liver free from haemoglobin exhibit a spectrum of the type exhibited by alkaline haematin. Consequently, the iron is present in the tervalent state. The following absorptions (in mμ) are found :

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[1] *Z. physiol. Chem.*, **1930, 192,** 171; **1931, 195, 39.**

I. 650	646–620	610	II. 550–530	520	510–490	463
	629		540		500	

According to Zeile the *inhibition* of the action of catalase *by hydrocyanic acid* depends on the production of a dissociable complex consisting of 1 mole of catalase haemin and 1 molecule of hydrocyanic acid. The inhibition by *hydrogen sulphide* is also due to the production of such a complex. These inhibitions are reversible—when the inhibitors are removed by suction, the inhibition disappears. *Carbon monoxide* has only a feeble, non-specific inhibitory effect. Wieland believes that the inactivation of catalase by hydrocyanic acid also depends on a non-specific action of this kind on the surface of the enzyme. He supposes that sometimes such action is the cause of the *sensitivity to hydrocyanic acid of respiration with oxygen.* If the destruction of hydrogen peroxide by hydrocyanic acid should be prevented then the production of this poison could proceed unchecked. (See Schardinger's enzyme, pp. 172 and 194.)

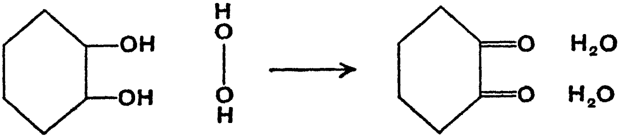
Haemin Catalysts

At this point it is appropriate to consider the catalytic power of the haemin-like active group. Numerous haemins exhibit catalytic activity, a property which is not possessed by the iron-free porphyrins. Haemoglobin is also able to act like catalase. Further, it has been found that haemin and haemoglobin exhibit peroxidase-like action (see p. 147). Finally, haemin can catalyse oxidations with oxygen such, for example, as those of linseed oil, fatty acids, glyceraldehyde and dihydroxyacetone. It is remarkable that those effects are *not sensitive* to the action of hydrocyanic acid (oxidase action). All these catalytic properties are encountered in the iron systems discussed in detail on pp. 145 *et seq.*, but in the iron systems they are less pronounced. The determining factors in the increased action of iron are its *specific state of combination* in the haemins (Warburg) and the *state of adsorption* of the haemin-like active constituent, for haemin adsorbed on animal charcoal has about twice the catalytic activity of non-adsorbed haemin (Kuhn). The following figures show how the factors mentioned above affect the action of iron :

1 mole (gramme-atom) of catalase iron decomposes 6×10^4 to 2×10^5 moles of H_2O_2 at 0°. The corresponding values for haemin and Fe^{II} or Fe^{III} are 0·01 mole (*i.e.* 10^7-fold less) and 0·00001 mole respectively.

2. Peroxidase

Peroxidase is the enzyme which can dehydrogenate quinone-producing compounds in the presence of hydrogen peroxide. Polyphenols, such as quinol, catechol, pyrogallol and also phenylenediamine, benzidine and leucomalachite green, are oxidised by hydrogen peroxide and peroxidase just as they are also by nitrite and hydriodic acid. It is possible that the peroxidases, in addition to catalase, serve for removing hydrogen peroxide from the cells. There is no difficulty in the way of regarding the peroxidases as dehydrogenases—they are able to activate the hydroxyl hydrogen of the polyphenols and to transfer it to hydrogen peroxide as acceptor. Hence the polyphenols are also dehydrogenases having pronounced donator and acceptor specificity (see p. 195).



In contrast to catalase, peroxidase is also able to act with monosubstituted peroxides, although more slowly. Mono-ethyl peroxide reacts five times more slowly than does hydrogen peroxide and peracetic acid reacts ten times more slowly.

The peroxidases usually occur within the cells. Most attention has been devoted to the vegetable peroxidases which are most abundant in roots and shoots. Animal peroxidases have been found in leucocytes, in the muscle of warm-blooded animals, in milk and so forth. They are best obtained from horse radish or from milk.

Up to the present the purest preparations obtained are those produced by Willstätter.

The *detection* of peroxidases depends on their capability to yield chromogen colours. For *quantitative determination* advantage is taken of several reactions of the peroxidases which are especially convenient and suitable for colorimetric comparison. In most cases these reactions depend on processes of so far unknown nature, the primary dehydrogenation of the quinone-producing compound being accompanied by all kinds of successive secondary reactions. Frequently these reactions are suitable for qualitative purposes only.

The following are used for *detection* :

(1) Nadi reagent : a solution of α-naphthol and dimethyl-*p*-phenylenediamine which gives a red colour with peroxidase and hydrogen peroxide.

(2) Tincture of guaiacum (Schönbein) : a solution of guaiacum resin in which the guaiaconic acid (constitution unknown) produces the blue colour.

In both cases caution is necessary since both these reagents are used for the detection of oxidases (see p. 228) and give colours when oxygen and oxidases are present. Therefore, a test without hydrogen peroxide should always be made.

(3) A series of other quinone-producing compounds, such as *o*-cresol, naphthol, pyrogallol, benzidine, may likewise be used.

For the determination of activity, three methods are available :

(1) Willstätter's pyrogallol method, described below.

(2) The malachite green method.

(3) The benzidine method.

Preparation of Peroxidase.[1]—Remove the damaged parts from 1 kg. of horse radish roots, leave them in water for a few hours, in order to make them suitable for slicing, and then cut into slices 0·5 to 1·0 mm. thick with a vegetable slicer. Subject the slices to dialysis in running tap-water for 4 to 6 days. (During the last days, the slices, especially those of material which gives a good yield, become dark yellow or brownish. Uniformly good results should be obtained with plants taken up to the middle of June.) Suck as dry as possible at the pump and digest for 4 to 6 hours with a solution of 8 g. of crystallised oxalic acid in 2 litres of water thus causing almost quantitative deposition of the peroxidase on the material of the cells so that stirring and grinding with water can extract only a small part. Filter with suction from the turbid acid liquid which contains much mustard oil, press down so that the weight is now only about 0·6 kg. and crush in a mill until a thin paste is obtained. Mix the paste with 1400 c.c. of water, filter with suction, using a large funnel and a cloth, and wash by drawing through the layer of material (which is about 3 cm. deep) a solution of 0·3 g. of oxalic acid in 3 litres of water. (The oxalic acid must be added in order to reduce the dissociation of the adsorption compound of peroxidase with the material of the plant.) Press down as dry as possible, first on the funnel and then in a press, repeatedly dividing up the cake formed in the cloth in the press, and re-pressing the pieces. Grind up the material finely (weight about 0·3 kg.), add *gradually* 200 c.c. of barium hydroxide solution (not quite half-saturated) and stir vigorously for ½-hour in order to avoid unequal distribution of the barium hydroxide and to prevent too much enzyme going into solution at this stage. (The fluid remains feebly acid or becomes neutral.) Press again and so obtain rather more than 200 c.c. of a preliminary extract containing a small amount of peroxidase.

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[1] Willstätter, *Annalen*, 1918, 416, 21. Only one-fifth of the amount of horse radish used by Willstätter is taken. Slice at right angles to the axis of the root.

To isolate the enzyme, grind the cake up again and stir with 250 c.c. of barium hydroxide solution saturated at 20°. After 45 minutes press well again so producing 330 c.c. of a very active extract. Transfer this at once to a stoppered flask and render faintly acid by shaking with carbon dioxide which precipitates only a small amount of barium carbonate, most of the barium hydroxide remaining absorbed in the vegetable matter. Prepare two more extracts by breaking up again the cake of pressed material and treating each time with 100 c.c. of half-saturated barium hydroxide solution (these extracts are not less pure than the first, but contain less peroxidase). Allow the hydroxide solution to act for 30 minutes.

Neutralise each of the extracts separately with carbon dioxide and add to each 90 per cent of its volume of 96 per cent alcohol producing thus a small, slimy precipitate but leaving the peroxidase in solution. Combine the extracts, leave in the refrigerator overnight to clarify and filter with suction through a layer of coarse talc on a cloth and obtain a clear filtrate. Concentrate this filtrate to 10–15 c.c. under reduced pressure during about 1½–2 hours maintaining the bath-temperature at 50° and the temperature in the flask at 30° and immersing the flask to the liquid level only.

Cool the receiver in an ice-salt mixture. Clarify the concentrated solution (turbid owing to the presence of some barium carbonate) by filtration with suction through a thin layer of talc, washing this subsequently with 5 c.c. of water used first to wash out the flask.

Precipitate the peroxidase then by adding, with shaking, five volumes of alcohol, which causes the enzyme to separate, first in the form of a few yellowish flocks, later as a dough-like mass at the bottom of the vessel. Leave for a short time in the refrigerator, and pour off the supernatant liquid, which contains but little enzyme, so as to avoid contamination with barium acetate, otherwise liable to crystallise out readily. Rub the dough-like material with alcohol, separate the powder so produced by filtration with suction and wash with alcohol. The material so obtained has a purpurogallin value of 130–260. If desired, carry the purification a little further by reprecipitation, preferably after addition of a very small amount of sulphuric acid in order to remove aluminium salts. Better still, purify by removing with mercuric chloride the glucosides which accompany the peroxidase. To obtain the most active enzyme adsorb on aluminium hydroxide and kaolin, and elute with carbonic acid.

The Pyrogallol Method.—Bach and Chodat took advantage of the production, from pyrogallol in the presence of peroxidase and hydrogen peroxide, of purpurogallin in order to determine the activity of peroxidase preparations. Their gravimetric method has the disadvantage that the high concentrations of hydrogen peroxide used, seriously damage the peroxidase. Willstätter[1] modified the method, greatly diluting the oxidising agent so that damage to the enzyme was avoided but employing sufficiently great excess of pyrogallol and hydrogen peroxide that the amounts of purpurogallin produced in equal times could be compared with each other, the amount of material transformed per unit of time being proportional to the amount of enzyme. The enzyme preparation, then, must be used in a concentration which is such that, at the most, the change in the concentration of the excess of substrate has practically no effect on the amount transformed during the experimental period. Thus, the conditions are : use of very dilute hydrogen peroxide in excess, which means use of large volumes of liquid and use of very great excess of pyrogallol. The purpurogallin is determined not gravimetrically but colorimetrically.

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[1] *Annalen,* **1918, 416, 44.**

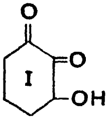
Procedure.—Dissolve 5 g. of purest pyrogallol with stirring in 2 litres of distilled water in a round-bottomed flask keeping the temperature constant at 20° and add about 10 c.c. of approximately 0·5 per cent hydrogen peroxide solution obtained from Merck's perhydrol by diluting 1 : 60. Take *exactly* 50 mg. of hydrogen peroxide as determined in advance by volumetric analysis. Add the *peroxidase* at the moment when the determination is commenced, using such an amount that in 5 minutes about 15–30 mg. of purpurogallin are produced. (As an indication : 0·25 mg. of the crude product prepared as described above, or a corresponding (smaller) amount of the purest material should be used.) For this purpose dissolve a small amount of enzyme, say 5 mg., in 100–500 c.c. of water and, with a pipette, transfer to the pyrogallol-hydrogen peroxide mixture the required aliquot portion thus producing an immediate yellow colour but (within the experimental period) no precipitate. After exactly 5 minutes (stop-watch) interrupt the reaction by adding 50 c.c. of pure, dilute, iron-free sulphuric acid. Immediately extract the liquid three or four times with ether. (The aqueous layer frequently retains a reddish colour due to the production of a small amount of by-product.) Compare the colour of the ethereal solution (volume 250–500 c.c.) in a colorimeter (see p. 20) with that of a standard containing 100 mg. per litre of purpurogallin recrystallised from alcohol and ether. The limit of error in reading the colorimeter amounts to ± 0·5 mg. of purpurogallin.

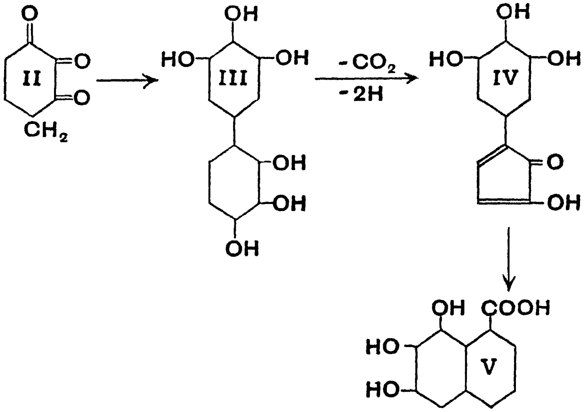
Thus, the method yields results accurate to within a few tenths of a milligramme of purpurogallin.

Determine the *purpurogallin value* (see below) of peroxidase material prepared as in the experiment described above.

Theory.—In the oxidation of pyrogallol in the presence of peroxidase and hydrogen peroxide or by oxidising agents there is produced a purple dye, *purpurogallin* which, as we have shown, can be determined by extraction with ether and colorimetric measurement. In a complicated reaction, not yet explained in all its details, the dye is produced in yields of up to 80 per cent of the pyrogallol consumed. It is very probable that other substances are produced at the same time.

Dehydrogenation produces first the unstable *ortho*-quinone (I) the tautomeric form of which, a triketo*cyclo*hexene (II), condenses with 1 mole of pyrogallol to give 2 : 3 : 4 : 3′ : 4′ : 5′-hexahydroxydiphenyl (III). This compound undergoes dehydrogenation (loss of 2H) and loses CO_2 and finally yields *purpurogallin* (IV), a bicyclic compound containing no condensed rings. Purpurogallin is very readily oxidised and can be determined only because of its low solubility. With alkali it yields purpurogallone (V), a genuine naphthalene derivative.





Definitions.—The *purpurogallin value* is that amount of purpurogallin which is produced in 5 minutes by 1 mg. of material in a mixture of 5 g. of pyrogallol and 50 mg. of H_2O_2 at 20°. The *peroxidase unit* corresponds to 1 mg. of enzymic material having purpurogallin value 1000. 5 g. of horse radish or 20 g. of turnips contain 1 peroxidase unit.

As to the *properties of peroxidase* it should be mentioned that the p_H optimum depends on the nature of the chromogen. For guaiacol the optimal p_H zone is 5·0–5·2. Peroxidase withstands relatively high temperatures. For example, the peroxidase of milk is not inactivated until the temperature of 80°–85° is reached, whilst the catalase of blood loses its activity on heating for 1 minute at 65°. *Hydrocyanic acid* and *hydrogen sulphide* but not *carbon monoxide* inhibit the action of peroxidase. 0·000005M-HCN reduces the activity by 50 per cent. The inhibition by hydrocyanic acid is not reversible. Hydrogen peroxide also in high concentrations inactivates peroxidase. The best peroxidase preparation so far obtained, namely, that of Willstätter and Pollinger,[1] had a purpurogallin value of 4900. According to Keilin, cytochrome (see below) and haematin from the interior of cells also have peroxidase action and are the cause of the peroxidase action of yeast cells which are free from peroxidase.

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[1] *Loc. cit.*

Soon after Zeile and Hellström had shown that catalase (see p. 206) contains a haemin complex, an analogous finding was made for peroxidase.[1] Until then it had been thought that no significance was to be attached to the iron present in peroxidase preparations as far, at least, as the peroxidase enzymic reaction was concerned, because as the degree of purity was increased the content of iron in non-specific combination steadily decreased and because haemin or iron itself exhibits only a fraction of the peroxidase action of peroxidase preparations. Actually, the content of active haemin iron is very low. Nevertheless, the haemin content is sufficient to confer on pure peroxidase preparations a light red colour. That the haemin complex is actually the active component follows from the fact that the haemin content, determined spectrophotometrically, of preparations of varying degrees of purity, runs parallel with the activity as measured by the purpurogallin value. From the absorption spectrum it may be concluded that the haemin concerned is ordinary protohaemin (blood-haemin) just as in the case of catalase. By using reducing agents and bases it is easy to produce, with peroxidase preparations, the typical haemochromogen spectrum (Fe^{II}) which exhibits especially distinct absorption at 557 and 527 $m\mu$ [2] (see p. 219 in connexion with cytochrome). Here also the method in which the haemin is combined in the molecule of the enzyme and the state of adsorption have important effects on the enzymic activity, for free haemin has only a millionth of the peroxidase activity of haemin in enzymic combination. It has been calculated that one gramme-atom of haemin iron in peroxidase can, under optimal conditions, reduce 10^5 moles of hydrogen peroxide per second. Since catalase (see p. 208) exhibits similar activity further evidence is provided of the presence in peroxidase of a haemin-like active component (Kuhn, Hand and Florkin). The investigations mentioned refer to vegetable peroxidase. Nothing is known about the active component of animal peroxidase but probably, as in the case of catalase, the active components of the animal and vegetable enzymes are identical.

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[1] Kuhn, Hand and Florkin, *Z. physiol. Chem.*, 1931, **201**, 255.

[2] Hand, *Ergebn. Enzymforsch.*, 1933, **2**, 272.

3. Cytochrome

In the year 1886 McMunn described a pigment occurring in animal cells which showed a characteristic absorption spectrum in the reduced state. This pigment he termed *myohaematin* and *histohaematin*. He succeeded also in isolating the components by extraction in the form of haemin compounds. However, Hoppe-Seyler vigorously contested McMunn's discovery, holding that he had isolated only transformation products of haemoglobin. In consequence, McMunn's work was forgotten.

But in 1925 Keilin[1] confirmed McMunn's observations and found that the pigment now termed *cytochrome*, consists of *three components* of the haemochromogen or parahaematin type and occurs throughout the whole domain of living matter from the warm-blooded animals to the unicellular vegetable organisms. It is never found in anaerobic organisms. The concentration of the pigment runs parallel with the intensity of respiration. Yeast, many aerobic bacteria, the breast-muscle of birds and so on are especially rich in cytochrome.

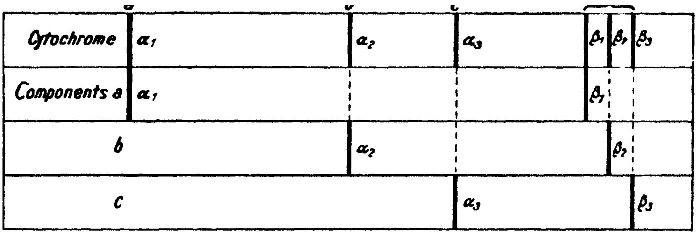

Fig. 31

Cytochrome in unicellular organisms or in tissues is detected by means of its typical *absorption spectrum*. The spectrum of *reduced* cytochrome is particularly characteristic. In the zone of visible light it shows four bands *a*, *b*, *c* and *d* which vary in position within very narrow limits for cytochromes from different organisms. In the case of cytochrome from bakers' yeast the bands are in the following positions—*a*, 6035 Å; *b*, 5645 Å; *c*, 5490 Å; *d*, 5190 Å. This spectrum, as will be shown in the description which follows, can very easily be observed, in a strongly illuminated suspension of yeast cells, with the help of a microspectroscope attached to a microscope. The spectrum of oxidised cytochrome, on the other hand, is not typical and is effaced. It resembles the spectrum of parahaematin. Of the four bands of reduced cytochrome (*a*, *b*, *c* and *d*), *a*, *b* and *c* are actually the α-bands of one cytochrome component, whilst the band *d* consists at least of β-bands of two cytochrome components (*B*- and *C*- components, see Fig. 31). The position of the β-bands of the component *A* cannot be given accurately. The discovery of the three components of cytochrome was made possible by the facts that the relative amounts of these in the various organisms vary, that the component *C* with its *c*- and *d*- bands can be extracted and so forth (see below).

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[1] *Proc. Roy. Soc.*, 1925, 98 B, 312.

As to the *production of cytochrome* it is probable that, like the other haemin-containing enzymes, it is derived from the free haematin present in all aerobic cells. According to Keilin the three haems of the cytochrome haemochromogens are different but the nitrogenous components, on the other hand, are identical. Of the three components, component *C*, which is stable towards heat, can be extracted and concentrated without losing activity and spectroscopic properties. Components *A* and *B*, on the other hand, are very easily destroyed.[1] The absorption bands of the porphyrin obtained from cytochrome-*C* lie between those of haemato- and proto-porphyrin. According to Zeile,[2] cytochrome-*C* has the fundamental porphyrin skeleton of the protoporphyrin type and the same arrangement of side chains as in the natural blood-pigment (aetioporphyrin III) which is condensed with a tertiary nitrogenous base.

Rôle of Cytochrome-*C* in the Cells.—Keilin took advantage of the fact that the spectrum of reduced cytochrome (Fe^{II}) is distinct and characteristic, whilst that of the oxidised form (Fe^{III}), on the other hand, can often hardly be observed in order to discover spectroscopically the conditions under which cytochrome is reduced and oxidised in the cells. A strongly illuminated suspension of bakers' yeast serves best for this purpose. It exhibits the spectrum of reduced cytochrome. If the suspension is shaken with air or if air is bubbled through it the reduced cytochrome is at once oxidised and the absorption bands disappear only to reappear, however, as soon as the oxygen is consumed. When an oxidisable substrate (a substrate capable of being dehydrogenated), such as glucose, is added to the cells or when the temperature is increased the reduction of the cytochrome is accelerated. On the other hand, the reduction can be retarded by reducing the amount of nutrient material in the cells, by decreasing the temperature and by adding *narcotics,* such as urethane, for example. Since narcotics inhibit the action of dehydrogenases, as can be observed by the methylene-blue technique or in respiration with oxygen, it is evident that in certain cases a *dehydrogenase* takes part in the process, this dehydrogenase reducing the oxidised cytochrome by means of the activated hydrogen of the substrate. On the other hand, the oxidation of cytochrome is inhibited by low concentrations of *hydrocyanic acid* (0·001*M*), hydrogen sulphide, hydrazoic acid and *carbon monoxide.* All these substances have a considerable inhibitory effect on *the respiration of cells.* According to Keilin they inhibit to the same extent the oxidation of *p*-phenylenediamine and the production by cells of indophenol from the Nadi reagent (see p. 209). These last reactions are attributed to the action of an oxygen-activating enzyme, namely, the so-called *indophenoloxidase* which, accordingly, Keilin introduces also into his scheme for the process of respiration —the process which likewise involves the oxidation of reduced cytochrome. Keilin's complete scheme therefore is

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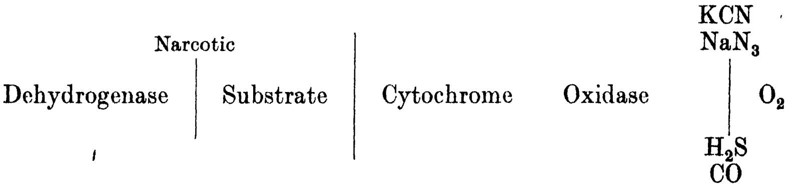
[1] Extraction of cytochrome-*C* see Keilin, *Proc. Roy. Soc.*, 1930, **106 B**, 418.

[2] *Z. physiol. Chem.*, 1933, **201**, 101.



The factors on the left side of this scheme have already been treated in detail on page 194. They constitute a dehydrogenase system of the Wieland and Thunberg type. The right half of the scheme, namely, cytochrome-oxidase-oxygen, corresponds, in its eventual action, to that of Warburg's respiratory enzyme (see p. 222). Keilin has suggested and constructed models in which both halves can be studied separately. The mode of action of the left half can be demonstrated with the system: dehydrogenase-substrate-methylene-blue-oxygen, methylene-blue acting as acceptor in the presence of oxygen but, as was already mentioned on p. 193, also as an oxygen carrier similar to that of the right half of the Keilin scheme. On the other hand, this can be demonstrated in a model experiment with indophenoloxidase isolated from cardiac muscle and cytochrome-*C* isolated from yeast. According to Harrison these together can oxidise cysteine with consumption of oxygen, the oxygen, activated by the indophenoloxidase, oxidising the reduced cytochrome which, by itself, is not auto-oxidisable and is reduced by cysteine. This system is sensitive to the action of hydrocyanic acid, carbon monoxide and hydrogen sulphide but not to that of narcotics.

The whole Keilin system then acts so that molecular oxygen is activated by indophenoloxidase and so made capable of oxidising the bivalent iron of the reduced cytochrome. The oxidised cytochrome is reduced by the hydrogen, activated by the dehydrogenase, of the substrate, the tervalent iron being converted into the bivalent (conversion of the parahaematin type into the haemochromogen type).

As to the so-called *oxidones*, said by Battelli and Stern to belong to the *indophenoloxidase group*, it should be pointed out that it has not yet been possible to separate these from the cells. According to these authors their distribution runs parallel with the magnitude of respiration of the cells, they are not able, alone, to oxidise carbohydrates, fats and so on, they are thermolabile and they are very sensitive to the action of cell poisons. Their behaviour towards *p*-phenylenediamine has been investigated. 1 g. of tissue was able to transfer, per hour, 9–12 c.c. of oxygen. Closely related to indophenoloxidase but, according to Keilin, not identical with it, is *potato-oxidase* which is widely distributed in the vegetable kingdom. Keilin concludes that indophenoloxidase only has all the properties required for the oxidase of his system. Since indophenoloxidase can be poisoned it may be assumed that it contains a heavy metal. It has not been discovered whether it contains a haemin complex as prosthetic group or what its relation is to Warburg's respiratory enzyme. It must not be forgotten that the indophenoloxidase is the weakest point in Keilin's scheme.

Experiment.—Study the spectral properties and mode of action of cytochrome, using bakers' yeast. For this experiment there are required a *micro-spectroscope*, a *microscope lamp* for alternating or direct current with a *resistance*[1] and a microscope with condenser.

Procedure.—Attach the micro-spectroscope to the microscope, switch on the light and adjust the beam so that it is reflected from the mirror of the microscope directly through the lens of the condenser. Prepare a suspension of bakers' yeast by stirring about 200–300 mg. with 5 c.c. of water. Transfer the suspension to a tall bottle. In order to cut off excess of light, stand the bottle on a cardboard lid having a circular opening about 1 cm. in diameter, placing this lid on a slide. Ensure that the mirror with the scale and mirror of the prism for comparison receive sufficient sunlight or artificial light to permit projection of the scale and of a spectrum for comparison. Before beginning the experiment, calibrate the scale with respect to the D line 589 mμ preferably by illuminating with sodium light. Make the layer of suspension of such thickness that the cytochrome spectrum can readily be seen.

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[1] Directions for use are provided with these pieces of apparatus.

1. After a short time the spectrum of reduced cytochrome with its characteristic lines first appears. When the bottle is shaken, *i.e.* when the suspension is shaken with oxygen the spectrum disappears at once but reappears after a short time. Oxidised cytochrome, the spectrum of which is not visible in this experiment, is reduced (according to Keilin) by the hydrogen of the donators as soon as the oxygen is consumed and the spectrum of reduced cytochrome appears.

2. In the same way a good cytochrome spectrum is obtained when a small piece of moist bakers' yeast is pressed between two slides and examined with the spectroscope.

3. When a small amount of an oxidising agent such as potassium ferricyanide or hydrogen peroxide is added to the suspension of yeast the cytochrome is likewise oxidised and the bands disappear.

4. Reducing agents such as quinol and hydrosulphite immediately reduce cytochrome even when the reducing power of the cells is diminished, say, by warming to 50°.

5. When 0·5 c.c. of 0·001M-potassium cyanide solution is added to the suspension, oxidation of the cytochrome is completely inhibited and the spectrum of reduced cytochrome persists even when the mixture is shaken with oxygen. Keilin explains this by supposing that oxidation of reduced cytochrome cannot occur when the indophenoloxidase is poisoned.

6. If a small piece of bakers' yeast is stirred with pyridine and a few small crystals of hydrosulphite it can be observed, even with the naked eye, that the suspension acquires a light red colour due to the production of haemochromogen. The protohaemin, which constitutes the bulk of the haemins of the cells, is the cause of this. Two typical absorption bands are observed, one at 556 and the other

at 520 mμ. The spectrum of the haemochromogen obtained from a mixture of chlorohaemin and pyridine by addition of hydrosulphite should be projected through the prism used for comparison and compared with that of the above haemochromogen. The chlorohaemin (a few small lumps of the crystalline product) is dissolved in pyridine and some hydrosulphite or quinol is added. Here also the two bands are at 556 and 520 mμ.

CATALYSIS BY HEAVY METALS AND ACTIVATION OF OXYGEN

It has already been explained in detail (see p. 145) that, in many cases, it is a matter of opinion whether one speaks of activation of hydrogen or activation of oxygen (that is to say, of dehydrogenase, oxidase or peroxidase action) in examples of catalytic oxidation by oxygen or hydrogen peroxide where a heavy metal acts as catalyst and hydrogen-containing material is oxidised. If the criteria for the occurrence of activation of hydrogen are the action of acceptors, the production of hydrogen peroxide and so on, then we must without doubt speak of activation of oxygen when production of peroxide is observed with heavy metal or substrate or when a substrate, activated by a dehydrogenase, is able to react with the usual acceptors indeed, but with oxygen only after a special catalyst containing heavy metal and adapted to the activation of molecular oxygen is present. Even in this last case one may be in doubt whether the catalyst containing heavy metal does not provide an additional hydrogen-activating effect. In other words, catalysis by heavy metal is not by any means the same thing as activation of oxygen.

When an enzymic reaction can be inhibited by substances, such as carbon monoxide, hydrocyanic acid and thiol compounds, *which form complexes*, this is taken as an indication that the enzyme *contains a heavy metal.* This criterion is not free from defects, for certain catalyses due to haemin are not inhibited by hydrocyanic acid (see p. 207). Also, catalase and peroxidase are not affected by carbon monoxide because only bivalent haemin iron reacts with this gas and in these enzymes, in contrast to the so-called respiratory enzyme (see p. 222), the iron is to be assumed to be tervalent. Accordingly, absence of inhibition by no means certainly indicates absence of heavy metal. The opinion that inhibition by hydrocyanic acid is due to non-specific blocking of the surface of the enzyme by the acid may be recalled here.[1] In the case of the dehydrogenases this can only occur when the oxidising agent is oxygen because of the great claims of the surfaces of the foreign acceptors, methylene-blue and quinone (see p. 193).

Peroxides have been isolated from the products of auto-oxidation of aldehydes and numerous organic compounds containing $C=C$ double bonds. The first stage in the auto-oxidation of aldehydes is the production of a per-acid. This process is accelerated by heavy metals.

$$\underset{H}{RCO} + O_2 = \underset{OOH}{RCO}$$

According to Wieland, however, this is not to be regarded as a general case of catalysis by heavy metals. Iron salts and haemin catalytically accelerate the auto-oxidation of unsaturated fatty acids, such as linolenic acid as well as that of lecithin and ergosterol, the peroxide being finally actually produced by way of intermediate products (Warburg, Wieland, Kuhn, Robinson). The primary addition of oxygen can be prevented by an *anti-oxidant*, for example, diphenylamine. For the rest there is no well-established conception of the mechanism of the process. According to Wieland, when iron peroxide plays a part reconversion to the ferrous stage must occur since only the ferrous form is active. General hypotheses concerning the process of auto-oxidation have been developed by Dufraisse and Moureu. Nothing is known about the existence of peroxides of the substrates in biological processes.

Manchot assumes the production, as a result of reactions induced by the ferrous ions, of a peroxide of the composition FeO_2 or Fe_2O_4. It is known that in the auto-oxidation of ferrous hydroxide by oxygen, arsenite simultaneously present is oxidised to arsenate. The ratio of the amounts of oxygen taken up respectively by the ferrous hydroxide and the arsenite, the induction factor, is 1 : 1 :

$$2Fe(OH)_2 + O_2 \longrightarrow 2FeO_2$$

$$2FeO_2 + Na_3AsO_3 + 3H_2O \longrightarrow 2Fe(OH)_3 + Na_3AsO_4.$$

Similar relations are encountered in oxidations with hydrogen peroxide induced by ferrous salt. But since, in many oxidations induced by iron, exact stoichiometrical relations are not found and the reactions rather assume the character of catalyses the mechanism of the reactions, as in the case of catalyses by iron (see p. 145), involves not such production of peroxide but rather that of ferrous-substrate complexes.

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[1] *Annalen*, 1928, 467, 127.

In many catalyses by iron, however, it suffices to assume a continuous alternation in the valency between ferrous and ferric iron (see p. 146). *Haemin catalyses* also involve such alternation of valency—for example, the conversion of haemoglobin into methaemoglobin and that of haem into haemin (see p. 198). The oxidation in the system methaemoglobin-activated carbohydrate occurs in this way, for example. These catalyses are of special interest in connexion with Warburg's *respiratory enzyme* in which, likewise, ferric iron is the oxidising agent.

In *peroxidase* the iron is present in the tervalent state. It is not known whether peroxide production and reduction to the bivalent state occur. In *catalase* also, the iron is tervalent. But here there is a difference not yet explained since, in model experiments, bivalent iron exhibits special catalase activity. According to Wieland both peroxidase and catalase can be regarded as specific dehydrogenases. If this is done, hydrogen-activating power is ascribed, for the first time, to the haemin catalysts of the cells. The view of Haber and Willstätter that the action of all dehydrogenases depends on heavy metal may also be justifiably applied here (see p. 229).

WARBURG'S VIEWS ON BIOLOGICAL OXIDATION

The theory of the biological *activation of oxygen* rests on the view that respiration is a form of *catalysis by heavy metal* and that iron, which occurs generally in the cells, is the metal concerned. According to Warburg, the *respiratory catalyst* is a substance like haemoglobin, the so-called *respiratory enzyme*, which is said to occur in all cells, animal and vegetable, which consume oxygen. This enzyme has nothing to do with the haemoglobin of blood. According to Warburg, respiration is a *surface reaction* and the respiratory enzyme is only active when in combination with the cells: it cannot be separated from them. The concentration of the respiratory enzyme in the cells is very low.

The *inhibition of respiration by hydrocyanic acid* takes place, in Warburg's view, as follows: the hydrocyanic acid adds itself to the tervalent iron of haemin and so suppresses the catalytic action of the respiratory enzyme. The inhibition of respiration by *carbon monoxide* is explained by the supposition that an addition product of the type of carbon monoxide-haemoglobin is produced. This compound is believed to be dissociable with the result that carbon monoxide and oxygen, in proportion to their partial pressures, compete for the respiratory enzyme.

The consumption of oxygen is supposed to occur thus: there is a continuous alternation between the ferrous and ferric stages and, consequently, transfer of oxygen takes place (see p. 146), the addition product first formed (oxidation product) being very rapidly converted by oxygen from the ferrous form (of the enzyme) into the ferric form (of the enzyme). This product then oxidises the substrate and is itself reconverted into the ferrous form.

In the presence of carbon monoxide only that portion of the respiratory enzyme which is not " blocked " provides for the uptake of oxygen. The carbon monoxide-respiratory enzyme compound is split by *light*, undergoing photo-chemical dissociation apparently because it absorbs light. Hence, if the respiration vessel containing oxygen and carbon monoxide is irradiated the inhibition is reversed. A possible method of showing that the respiratory enzyme is a haemin compound is thus provided. A numerical relation can be demonstrated between the action of light and the absorption of light by the carbon monoxide compound; the absorption of the carbon monoxide compound cannot be measured directly, however, for the concentration of the enzyme in the cell (4×10^{-7} to 3×10^{-8} g. of Fe per g. of dry material) is too low. Accordingly, Warburg,[1] instead of determining the absorption spectrum of the respiratory enzyme, determines the so-called "*action spectrum*" which is proportional to the absorption spectrum. Only absorbed light is photo-chemically active. Hence, if a suspension of cells in the presence of a mixture of carbon monoxide and oxygen is irradiated with *light of various wave-lengths* the respiration will be increased in varying degrees on account of the dissociation of the carbon monoxide compound of the respiratory enzyme. The action spectrum, and hence also the absorption spectrum of the compound, can be deduced from the observed changes in the magnitude of the respiration. Such measurements have been made on yeast, acetic bacteria, the retina and so on. The validity of the method has been tested with models, such as, for example, carbon monoxide-haemochromogen, carbon monoxide-ferrocysteine, and so forth. The *law of photo-chemical equivalence* according to which 1 quantum of light splits 1 molecule of carbon monoxide compound usually holds. The spectrum of the carbon monoxide compound of the respiratory enzyme is shown in Fig. 32.

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[1] Warburg and Negelein, *Biochem. Z.*, 1928, 193, 339; 1929, 202, 202; 1929, 204, 495; 1929, 214, 64.

As far as the nature of the haemin component is concerned all that is known has been derived from comparative measurements of the absorptions of other haemoglobins. Certain so-called phaeohaemins, such as, for example, spirographis-haemin (from the *Spirographis* worms of the Mediterranean) and cryptohaemin (pigeon) which are very closely related to the haemin of the enzyme, are known. A comparison of the absorption maxima of the CO-compounds of some of these haemins is given below (in mμ):

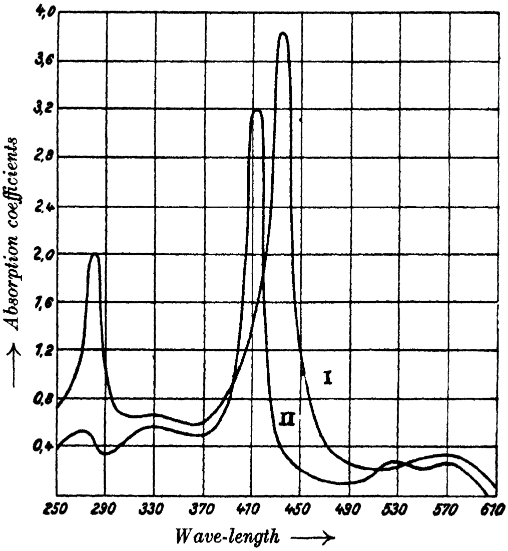


FIG. 32

I. Absolute absorption spectrum of the CO-compound of the respiratory enzyme.
II. Absorption spectrum of CO-protohæmin.

CO-Compound of respiratory enzyme	590	433
CO-Compound of the haemoglobin of blood	570	420
CO-Compound of spirographis-haemin	594	434

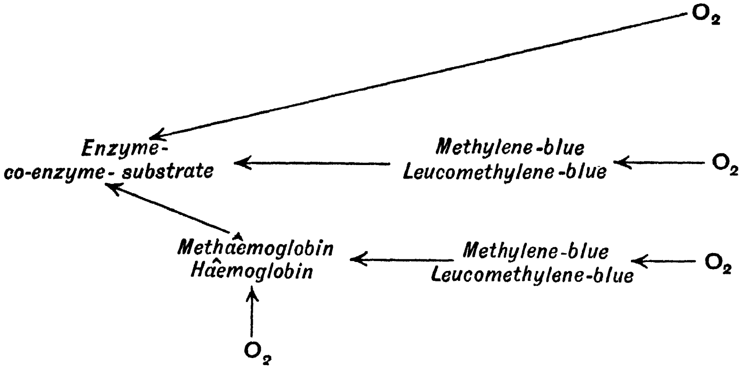
According to Warburg, the respiratory enzyme is a non-specific oxygen carrier. But it must be assumed that selection may take place from amongst the substrates which are available for oxidation. In the attempt to bring this conception into harmony with the dehydrogenation theory the existence of various dehydrogenases is assumed. Also, it has been found that, *in vitro*, physiological " combustibles ", such as sugar, for example, are not oxidised by tervalent haemin iron, whereas, apparently, this form of iron in the cells does oxidise these " combustibles ". Warburg, therefore, has more recently made the assumption that the oxidisable material of the cells must be prepared for combustion. He terms this process *activation of the substrate*—a process which is equivalent to the activation of the hydrogen of the substrate of Wieland's theory. Warburg's extended scheme then is :

Activation of the oxidisable material.	Activation of oxygen by the respiratory enzyme.

This two-stage scheme corresponds closely to that of Keilin (see p. 217) especially since, very recently, Warburg has introduced into his scheme the carrier effects of the components of cytochrome. According to Warburg the activation of the substrate is the process which determines the rate of respiration with oxygen because the enzymic iron, which transfers the oxygen, is in excess. An enzymic system of this kind which activates hexose monophosphate (Robison's ester) is present, as Warburg has shown, in the blood corpuscles. It consists of enzyme and co-enzyme which also can be obtained separately—the first from the corpuscles of rats' blood or yeast and the second from the corpuscles of horses' blood. This enzymic system corresponds to a dehydrogenase system.

The scheme given below shows the mechanisms in which this substrate-activating enzymic system can play a part, thus :

The hexose phosphate activated by the enzymic system can be directly oxidised by oxygen or by methylene-blue as well as by oxygen with methylene-blue as carrier. With methaemoglobin, which is reduced during the process to haemoglobin, acetaldehyde and carbon dioxide are produced. If oxygen is present at the same time the haemoglobin is reoxidised to methaemoglobin and so acts as a carrier-catalyst. If, in addition, methylene-blue is added, oxygen is taken up because the methylene-blue oxidises haemoglobin to methaemoglobin, being itself thereby reduced to leucomethylene-blue and again oxidised by oxygen.



According to Warburg the direct interaction of methylene-blue with activated hydrogen and the indirect interaction by way of haemin iron occur also with the cell itself (intact or cytolysed blood-cells, acetic bacteria). Respiration with methylene-blue and with quinone is inhibited, although to a very small extent only, by carbon monoxide and hydrocyanic acid (see p. 193). This is explained by supposing that, according to the concentration of the methylene-blue or quinone, there are two courses of action open to these two acceptors. Only the route which passes by way of haemin iron can be blocked—not the other. In the last case, then, the respiratory enzyme cannot play a part. The same holds for respiration with oxygen only a fraction of which is ordinarily unaffected by hydrocyanic acid. The biological oxidations which occur, for example, in *Chlorella* (algae), press-juice from yeast and facultative anaerobic bacteria, such as *B. Delbrückii* and *B. acidophilus* (Bertho), are completely unaffected by hydrocyanic acid and carbon monoxide because these oxidations do not depend on the co-operation of haemin compounds.

As has already been pointed out on p. 166, these catalase-free *lactic acid bacteria* oxidise glucose and also the C_3- and C_2-compounds produced in the degradation of sugars, namely, dihydroxyacetone, methylglyoxal and so on, with production of carbon dioxide, all the oxygen consumed being recovered as *hydrogen peroxide*.[1] The *respiratory quotient* of this respiratory process CO_2/O_2 is therefore

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[1] Bertho and Glück, *Naturwiss.*, 1931, 19, 88; *Annalen*, 1932, 494, 159.

0·5. Since these bacteria are quite free from haemin compounds this is a perfectly clear case of dehydrogenation.

Warburg and Christian,[1] however, some time later, attributed the hydrogen peroxide production just mentioned to the action of an *intermediate catalyst* free from heavy metal, which they detected spectroscopically in these very bacteria as well as in yeast and in a series of other organisms and were able to isolate from extract (Lebedev) of bottom yeast. This so-called "*yellow enzyme*" is able, like methylene-blue, to act as an oxygen carrier, being reduced by activated hydrogen to a colourless leuco-form and reoxidised by oxygen with production of hydrogen peroxide to the yellow form. According to these authors the "yellow enzyme" acts alike *in vivo* and *in vitro* in the system :

$$\begin{array}{l} \text{Hexose mono-} \\ \text{phosphate} \\ \text{(Robison's} \\ \text{ester)} \end{array} \left\{ \begin{array}{l} \text{Enzyme (from yeast)} \\ \text{plus co-enzyme} \\ \text{(from horses' blood)} \\ \text{(substrate-activating} \\ \text{system)} \end{array} \right. \longleftarrow \text{“ yellow enzyme ”} \longleftarrow O_2$$

and is said to transfer the whole of the oxygen during the respiration of the bacteria mentioned which contain 115 mg. of the pigment per kg. of dry material. Quite apart from the fact that with the isolated components of the system no evolution of carbon dioxide can be observed, it may be shown (Bertho and von Zychlinski [2]) that the above system, in contrast to the living bacterium, is not able to cause respiration with such respiratory substrates as glucose, dihydroxyacetone and so on, with production of carbon dioxide and hydrogen peroxide. Since it has not yet been shown with certainty that aerobic and anaerobic dehydrogenases can be activated by the "yellow enzyme" with consumption of oxygen, it is scarcely probable that other hydrogen-activating systems together with the "yellow enzyme" are concerned in the respiration of these or other substrates. Hence the assumption cannot be forthwith rejected that, in the respiration of the lactic acid bacteria, there is direct interaction of activated hydrogen and molecular oxygen.

In the respiration of *yeast*, which contains haemin compounds, the "yellow enzyme" plays no important part and might easily account for that small part of the residual respiration which is resistant to hydrocyanic acid. Occasionally, however (as in the respiration of *the liver of the rat*), it may act like cytochrome (see p. 215) as a carrier-catalyst, the haemin compounds taking part at the same time. It seems as if the " yellow enzyme " is an important factor rather in the dismutation processes of fermentation than in the respiratory processes of yeast. Its activity as an intermediate catalyst in anaerobic dehydrogenations also harmonises with this view (Wagner-Jauregg).

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[1] *Naturwiss.*, **1932, 20, 688, 980**; *Biochem. Z.*, **1932, 254, 438; 1933, 260, 499; 1933, 266, 377.**

[2] *Annalen*, **1934, 512, 81.**

The " yellow enzyme " belongs to the class of *flavins* or *lyochromes*. These are greenish-yellow, water-soluble pigments which are widely distributed in the animal and vegetable kingdoms, for example, in yeast and liver (hepatoflavin) in which they are bound to protein-like carriers of high molecular weight and in eggs (ovoflavin), cows' milk (lactoflavin) and urine (uroflavin) in which they occur free (Kuhn and co-workers ; Ellinger and Koschara). The pigmentary components of the flavins and flavoproteins (they are named according to their sources) are frequently identical. This holds, for example, for the flavins of yeast, liver and grass, which are all identical with lactoflavin. Lactoflavin has the constitutional formula shown on p. 70. It is 6 : 7-dimethyl-9(*d*-ribityl)-*iso*alloxazine.

The " yellow enzyme ", also called *flavin enzyme*, was the first flavin of high molecular weight to be isolated.[1] It loses its activity as soon as the flavin component is separated from the protein residue. Free flavins, however, can act as hydrogen acceptors (Wagner-Jauregg). Lactoflavin is identical with vitamin–B_2 and, without doubt, the precursor (pro-enzyme) of the flavin enzyme which is produced in the animal body by the union of the flavin component with protein, and phosphoric acid can be regenerated from flavinphosphoric acid and the protein component (Theorell). Hence it is very probable that its vitamin action is related to the part which it plays in enzymic (desmolytic) processes. The absence from the diet of vitamin–B_2 (like that of the other B–vitamins, *e.g.* the anti-beriberi vitamin B_1) results in cessation of growth and specific symptoms of deficiency. Lack of vitamin–B_2 easily causes skin diseases. The effects of this vitamin are tested with rats.

There exist, in nature, simple oxidation systems in addition to those of the haemin oxidases so far discussed. These are the systems of the so-called *simple oxidases.* All that is known about the active component of these is that it contains a heavy metal. This is deduced from the inhibition produced by hydrocyanic acid. The simple oxidases are enzymes of the indophenoloxidase type (see above). To them is ascribed capability to activate oxygen and they are able to oxidise cyclic chromogens (*o*-dihydric phenols, such as catechol, adrenalin and so on, for example) with production of dyes. Whether or not these *phenoloxidases* are to be regarded as dehydrogenases is a matter of opinion. *Tyrosinase* excepted, they do not show any particular degree of specificity. Colour reactions such as those with guaiacum tincture, benzidine, *p*-phenylenediamine and Nadi reagent serve for detecting them.

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[1] *Biochem. Z.*, 1932, 254, 438.

THE THEORY OF HABER AND WILLSTÄTTER

In this general theory of biological oxidation, as in the theory of Wieland, the elimination of hydrogen from the biological substrate is the predominating factor in the mechanism of the reaction.

With regard to experiments, the object of which is to discover whether or not biological oxidations are to be regarded as reactions catalysed by iron, this theory attributes a predominating rôle to iron. In the dehydrogenation theory no decision is made as to whether or not the dehydrogenases contain heavy metal. It seems also that, in general, they do not.

According to Haber and Willstätter (contrast with Wieland's view) the elimination of hydrogen from the substrate takes place at first with simultaneous monovalent reduction of the heavy metal of the dehydrogenase. The hydrogen atoms are not eliminated in pairs just as the successive reactions involved in synthesis do not involve transfer of pairs of hydrogen atoms.

In the development of the theory the auto-oxidation of the alkali sulphites was taken as an example. Here, as in organic and enzymic reactions, the sulphite-ion SO_3'' is first oxidised by the catalyst to the radical SO_3H, the process being univalent since the catalyst acts in this way. With the cupric ion $Cu^{\cdot\cdot}$ as an ion having a univalent action the scheme is :

$$SO_3'' + Cu^{\cdot\cdot} + H_2O = SO_3H + Cu^{\cdot} + OH' \quad . \quad . \quad . \quad (I)$$

If the dimerisation of two non-paired monothionic acid radicals is neglected and the possibilities for interaction of these radicals with paired dissolved substances are rather considered then, according to Frank and Haber, when oxygen is also present the following are the two chief reactions :

$$\underset{\downarrow}{\mathrm{SO_3H}} + \mathrm{O_2} + \mathrm{H_2O} + \mathrm{SO_3}'' = 2\mathrm{SO_4}'' + \underset{\downarrow}{\mathrm{OH}} + 2\mathrm{H} \quad . \quad . \quad . \quad (\mathrm{II})$$

$$\underset{\downarrow}{\mathrm{OH}} + \mathrm{SO_3}'' + \mathrm{H}^{\cdot} = \mathrm{OH}' + \underset{\downarrow}{\mathrm{SO_3H}} \quad . \quad . \quad . \quad (\mathrm{III})$$

The monothionic acid radical produces the non-charged hydroxyl radical (II) which, in its turn, again produces the monothionic acid radical. By alternation of the two main reactions (II) and (III), a series of successive reactions is produced as is required by investigations of another kind (Christiansen, Bäckström). The univalent reduced catalyst, the cuprous ion, undergoes auto-oxidation and can initiate a new series of reactions. There is, in addition, a slight possibility that the OH- and SO_3H-radicals may dimerise, giving hydrogen peroxide and dithionic acid, or may interact with each other giving sulphuric acid. This then leads to temporary interruption of the succession of reactions. (For details, especially the physico-chemical, see *Ber.*, 1931, **64** [**B**], 2844.)

Organic and especially enzymic processes proceed in the same way. The application to the case of acetaldehyde will now be developed.

The catalyst, heavy metal or enzyme containing heavy metal, undergoes univalent reduction (ferric→ferrous). The substrate loses 1 H-atom undergoing univalent oxidation. The free valency so produced may be one of the carbon or one of the oxygen valencies. In the biological oxidation of acetaldehyde to acetic acid, the free valency, as may be concluded from the production of pinacones in the reduction of ketones, is one of those of carbon. The initial reaction, then, proceeds as follows :

$$\mathrm{CH_3{\cdot}CHO} + \mathrm{Enzyme} = \underset{\downarrow}{\mathrm{CH_3{\cdot}CO}} + \mathrm{Monodeoxyenzyme} + \mathrm{H}^{\cdot} \quad . \quad (\mathrm{I}a)$$

The subsequent reactions are then analogous to the two outlined for the inorganic example, namely, (II) and (III), continuous repetition of two main reactions, (II*a*) and (III*a*) occurring :

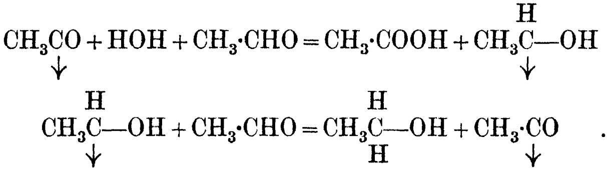
$$\underset{\downarrow}{\mathrm{CH_3{\cdot}CO}} + \mathrm{CH_3{\cdot}CHO} + \mathrm{O_2} + \mathrm{H_2O} = 2\mathrm{CH_3{\cdot}COOH} + \underset{\downarrow}{\mathrm{OH}} + \mathrm{H_2O} \quad (\mathrm{II}a)$$

$$\underset{\downarrow}{\mathrm{OH}} + \mathrm{CH_3{\cdot}CHO} = \underset{\downarrow}{\mathrm{CH_3{\cdot}CO}} + \mathrm{H_2O} \quad . \quad . \quad (\mathrm{III}a)$$

For an account of the formulation of intermediate peroxides the original literature must be consulted.

The reaction characteristic of the mechanism of aerobic processes caused by an oxidase (= dehydrogenase) is the main reaction (II*a*) not the initial reaction (I*a*). This initial reaction, and hence the radicals produced by dehydrogenation, persist even in the dismutation of acetaldehyde with the co-operation of water or in the dehydrogenation of acetaldehyde by hydrogen acceptors, such as quinone and methylene-blue. Thus Wieland's view of the identity of dehydrogenase (= oxidase) and mutase is accepted. Anaerobic dismutations and dehydrogenations by so-called hydrogen acceptors differ from aerobic processes in that, as the following equations (II*b*), (III*b*), (II*c*) and (III*c*) show, an organic radical takes the place of the free hydroxyl group.

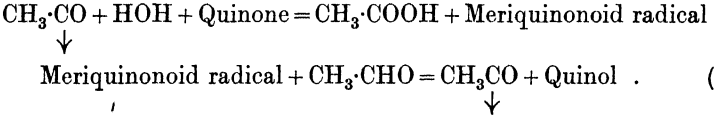
Dismutation : introductory reaction (I*a*) (see above) :



$$\underset{\downarrow}{CH_3CO} + HOH + CH_3\cdot CHO = CH_3\cdot COOH + CH_3\overset{H}{\underset{\downarrow}{C}}\text{—}OH \qquad (IIb)$$

$$CH_3\overset{H}{\underset{\downarrow}{C}}\text{—}OH + CH_3\cdot CHO = CH_3\overset{H}{\underset{H}{C}}\text{—}OH + CH_3\cdot \underset{\downarrow}{CO} \quad . \qquad (IIIb)$$

Dehydrogenation by quinone: introductory reaction (1*a*) (see above) :



$$CH_3\cdot \underset{\downarrow}{CO} + HOH + \text{Quinone} = CH_3\cdot COOH + \text{Meriquinonoid radical} \qquad (IIc)$$

$$\text{Meriquinonoid radical} + CH_3\cdot CHO = CH_3\underset{\downarrow}{CO} + \text{Quinol} \quad . \qquad (IIIc)$$

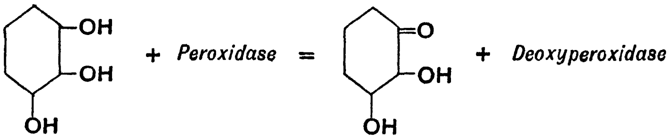
In this last case a meriquinonoid appears as the second radical.

The *re-formation of the enzyme* from the mono-deoxyenzyme must also be briefly considered here. In aerobic processes it is mainly a question of simple auto-oxidation. In anaerobic processes the re-oxidation could be brought about by the hydrogen acceptor present, for example, quinone. For other possibilities, see the original literature.

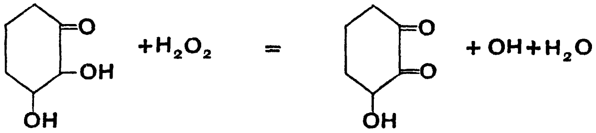
As in the inorganic example, the chain of reactions can be interrupted by dimerisation of the two kinds of radicals present, by interaction of these radicals or else by exchange of hydrogen to produce stable compounds. Of the possibilities discussed for the above examples, the most interesting in the present connexion are the combination of two hydroxyl radicals to give *hydrogen peroxide* and also production of *acetoin* which is actually observed during the dismutation of acetaldehyde by biological material. The above scheme fails to account for the cases where the oxygen is practically completely recovered as hydrogen peroxide and also, sometimes, for such cases where it is extremely questionable whether the dehydrogenase contains heavy metal (lack of sensitivity towards the action of hydrocyanic acid, see p. 226). The scheme can naturally be applied equally well to the explanation of intramolecular dismutations and also to that of the transfers of hydrogen which are observed during the decomposition of the sugar molecule by desmolysing enzymes during fermentation and glycolysis.

The mode of action of peroxidase and catalase can also be explained by this scheme. In the light of the theory of dehydrogenation both enzymes appear as dehydrogenases having especially pronounced donator and acceptor specificity (see pp. 201 and 208). This conception is also accepted in the present case and hence the formulations concerned closely resemble those given above.

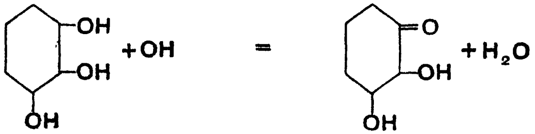
Peroxidase action :



(I*d*)



(II*d*)



(III*d*)

Catalase action :

H_2O_2 + Catalase = O_2H + Deoxycatalase . . . . (I*e*)

O_2H + H_2O_2 = O_2 + OH + H_2O . . . . (II*e*)

OH + H_2O_2 = H_2O + O_2H . . . . . . (III*e*)

Although these enzymes in particular, because of their iron content, appear best suited to fulfil the conditions for the application of the theory, serious difficulties are nevertheless encountered in attempting to explain the specificity of the two enzymes. According to the two schemes, it does not necessarily follow that peroxidase does not also exhibit catalase action, and catalase, on its part, peroxidase and dehydrogenase actions, for in both cases the transformations are brought about by OH-radicals not differentiated in any way. For a suggestion which explains the special behaviour of peroxidase see the original literature.

A mechanism involving successive reactions of univalent radicals may also be assumed in the case of many purely organic reactions catalysed by metals, especially iron.

Beyond doubt the theory of Haber and Willstätter represents progress in the combination of the two main theories, although it must be remarked that it has not yet sufficed to explain, without contradiction, a series of experimentally determined facts (specificity relationships, production of hydrogen peroxide, lack of heavy metal).

III. GLYCOLYSIS

ALCOHOLIC FERMENTATION

Glycolysis is the term applied to the enzymic breakdown of the carbon chain of the sugars under anaerobic conditions, that is to say, in the absence of oxygen. Hence all anaerobic fermentations and the anaerobic degradation of carbohydrates in animal cells are glycolytic processes. The term glycolysis is, in general, applied to the production of lactic acid in the animal organism.

Leeuwenhoek, Gagniard-Latour and especially Schwann earned the credit for the scientific discovery that the yeast fungus (*Saccharomyces*) is the vegetable germ organism necessary for bringing about the process of fermentation. Whilst Liebig denied the significance of this micro-organism for the fermentation process and to the end defended his chemical decomposition theory, the newly originated biological view grew up with Pasteur as leading exponent. This purely vitalistic view provided no explanation of the enzymic processes caused by yeast. E. Buchner showed that the enzymic actions of yeast can be separated from the life processes.

The classical equation for fermentation is:

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2.$$

SUGAR PHOSPHATES

In fermentation and glycolysis, the enzymic processes of cleavage of carbohydrates in vegetable and animal cells, phosphoric esters of the carbohydrates are presumed to be intermediate products. Therefore, these esters are the subject of detailed chemical and biological investigation. Since they are easily converted, the one into the other, a place can easily be found for them in the various theories of glycolysis and fermentation. In the case of yeast it has not yet been possible at all to study the transformation of these esters in the intact cell; in the case of muscle it has been possible but only incompletely. Accordingly, solutions of enzymes and stable preparations of cells (see pp. 248 and 264) have been used in order to follow the transformations of the phosphates of the carbohydrates. Since, however, the harmony of the reactions as they occur in the cells is not maintained in solutions of the enzymes, the use of these solutions is only an expedient.

A brief account of the most important sugar phosphates will now be given. In the main, these are named after their discoverers.

In determining and characterising the hexose phosphates, Bertrand's solution (see p. 74), which is reduced both by aldoses and ketoses, is used. The hypo-iodite reaction (Willstätter and Schudel, see p. 76) serves for the determination of the aldoses. In addition, salts are analysed and optical rotation and rates of hydrolysis are measured.

1. **Harden and Young's Ester.**—In the year 1909 these workers showed that one and the same hexose diphosphate is produced in the fermentation of fructose, glucose and mannose. The chief component is fructose, since the reducing power, as measured with alkaline hypo-iodite, is very small. This ester may be regarded as a 1 : 6-fructose diphosphate having a butylene oxide ring (formula I below).

2. **Neuberg's Ester.**—This is produced from Harden and Young's ester by elimination of the phosphate residue at C_1.

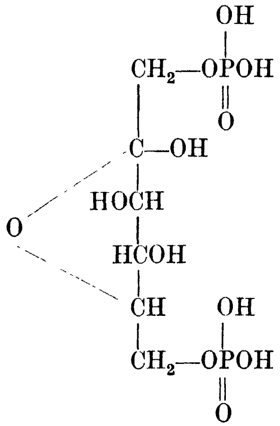
3. **Robison's Ester.**—This, likewise, is a monophosphate. It is obtained together with Harden and Young's ester from fermenting yeast maceration-juice. But like all the other sugar phosphates it is not a pure substance but contains, as shown by determination of its reducing power, about one-fifth of its weight of ketose and also glucose (II). This conclusion is reached by determining its re-

ducing power by Bertrand's method (aldose plus ketose) and its aldose content by Willstätter and Schudel's method (aldose only).

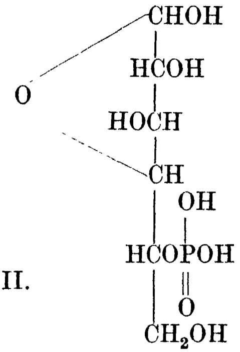
4. **Embden's Ester.**—This was originally called lactacidogen. It is, in the main, identical with Robison's ester and is obtained from rabbit muscle.

In addition to the four esters mentioned, there is a trehalose monophosphate obtained together with esters 1 and 3 during fermentation with dried yeast or zymin, and from muscle, two other hexose diphosphates of unknown constitution (Lohmann):

I.



II.



Theory of the Transformation of Hexose Phosphates

The first phases of the degradation of sugar are to a large extent the same in both alcoholic fermentation and lactic acid production.

In both cases the following holds essentially:

1. Inorganic phosphate must be present. It is esterified during the course of the breakdown of the sugar.
2. The same hexose phosphates are produced, namely, Robison's or Embden's ester and Harden and Young's ester.
3. A *co-enzyme* co-operates. The co-enzyme in fermentation by yeast, however, is different from that involved in production of lactic acid.
4. Arsenate accelerates and fluoride inhibits lactic acid production and fermentation.

In the year 1905 Harden and Young instituted fermentation experiments with Buchner's yeast press-juice, some without addition of phosphate, some with the addition of varying amounts. They found that press-juice from yeast alone had only 2–5 per cent of the fermenting power of living yeast, whereas, when suitable amounts of phosphate were added the value rose to 50 per cent. Further, they discovered that in the first, rapid phase of fermentation the rate at which the free phosphate was bound was the same as that at which the fermentation—evolution of carbon dioxide—proceeded. The phosphoric acid was used up with production of zymophosphates—hexose phosphoric esters—and the ratio CO_2 : phosphate esterified was equal to 1 as already mentioned. The first of these zymophosphates to be isolated was that named after its discoverers—Harden and Young's zymodiphosphate, γ-fructose-1 : 6-diphosphate. When all the phosphate was bound the rate of fermentation decreased considerably, and, in the second, slow phase of fermentation the diphosphate was dephosphorylated.

According to Harden and Young the *first, rapid* phase of the fermentation should be expressed by the following equation. A phosphorylating enzyme, namely, *phosphatese* takes part in the process, but, in general, it is difficult to separate it :

$$\text{I. } 2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_5OH + 2H_2O + C_6H_{10}O_4(PO_4R_2)_2.$$

In the *second, slow phase* of the fermentation the phosphate is again split off by means of the enzyme *phosphatase.* In this process fermentable sugar is re-formed (it can be isolated as fructose according to Harden and Young) and reacts again as in equation I :

$$\text{II. } C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2PO_4HR_2.$$

In the year 1914 Robison isolated from fermenting yeast juice another phosphoric ester which proved to be a monophosphate. The isolation of this ester raised a difficulty for the explanation of the process of fermentation because the ratio $CO_2 : PO_4 = 1$ is not affected by the phosphorylation of the mono- to the di-phosphate. Both esters are produced at the same time and in greatly varying proportions. The following equation, which is based on equation I, expresses the course of the fermentation when monophosphate is produced :

$$\text{I}a.\ 3C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_5OH + 2C_6H_{11}O_5(PO_4R_2).$$

Meyerhof and Lohmann showed by experiment how the mono-ester could be included in the equation expressing the course of fermentation in yeast juice and muscle extracts. They showed that, in the breakdown of sugar, the first reaction product is a mono-ester not identical with the Embden or Robison ester. These mono-esters can be regarded as stable but nevertheless very reactive products, and in fact they are much more slowly fermented than is free glucose. It is more probable that a more labile mono-ester should be produced (III*a*) and that this ester should undergo spontaneous stabilisation in the form of Robison's ester. Of 2 molecules of the latter 1 will be fermented, whilst the other is transformed into hexose diphosphate (III*b*). Thus Meyerhof and Lohmann reach a *new method of formulation* which also holds for lactic acid production in muscle extract :

$$\text{III}a.\quad 2C_6H_{12}O_6 + \underline{2R_2HPO_4} = 2C_6H_{11}O_5PO_4R_2{}^+ \text{ (active)} + 2H_2O.$$

$$\text{III}b.\quad 2C_6H_{11}O_5PO_4R_2{}^+ = \underline{2CO_2} + 2C_2H_5OH + C_6H_{10}O_4(PO_4R_2)_2 + H_2O.$$

According to Meyerhof, however, direct fermentation of zymophosphate may also occur :

$$\text{IV.}\quad C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = 2CO_2 + 2C_2H_5OH + 2R_2HPO_4.$$

This follows from the stimulation of the fermentation by *arsenate* which, as can be shown, does not stimulate the phosphatase itself when isolated as far as possible (this phosphatase is the dephosphorylising enzyme of equation II), whilst it none the less causes increased liberation of phosphoric acid from diphosphate and evolution of carbon dioxide in yeast press-juice. Consequently, arsenate does not stimulate the simple hydrolysis of the zymophosphate by phosphatase according to equation II but does stimulate direct fermentation of the diphosphate as in equation IV.

In contrast to this, the fermentation is inhibited by *sodium fluoride*. Sodium fluoride specifically inhibits the action of the phosphatase of yeast, the dephosphorylising enzyme (Lipman), whilst it has no effect on kidney phosphatase (Auhagen).

The equations given hold for press-juice and maceration-juice from yeast, as well as for muscle extract, but are less satisfactory for dried yeast and yeast dehydrated with acetone. They cannot be applied without alteration to express the processes occurring in intact muscle since Harden and Young's ester has never yet been detected in it. With dry bottom yeast large amounts of sugar phosphates are accumulated; but not with top yeast. The two Harden and Young equations (I and II) also hold, however, as von Euler and Johannsen have shown, for the fermentation, under certain conditions, of glucose by *living* yeast in the presence of phosphate over a large part of the course of the fermentation.

Fermenting living yeast, however, normally has only very small amounts of phosphate at its disposal, namely, the amounts which are present in the cell itself. During *natural* fermentation, hexose diphosphate is not accumulated. Thus, it is perfectly justifiable to raise the question: Why does living yeast require no transformation of phosphate, whilst yeast press-juice, on the other hand, does require it in order to cause a similar degree of fermentation? The general view is that phosphorylation must also occur of necessity in the living yeast cell and that this phosphorylation is no secondary reaction, the more so as in carbohydrate metabolism also, *i.e.* in the degradation of glycogen in muscle, liver and blood-corpuscles, hexose phosphate must be produced (Lohmann's two diphosphates, Embden's lactacidogen).

According to Harden, the accumulation of hexosediphosphate during the transformation of phosphates in the press-juice of yeast should probably be traced to the fact that in the juice the normal cycle of reactions which occur in living yeast, is disturbed, and, further, that in the living material the regulatory activities of the cells continuously provide the necessary optimal amount of inorganic phosphate in biological form. The Harden and Young ester and the two mono-esters when compared with the hexoses are not appreciably fermented by living yeast.

Whilst yeast preparations are most active at p_H 6·2–6·6—this is also the optimal p_H range for the enzymic production of hexose diphosphate—living yeast has the very wide (chiefly acid) zone between p_H 2 and 8 for its optimal effects. This phenomenon is often observed in living cells and is probably connected with the fact that cells can often produce, in their interior, a p_H other than that of the surrounding solution.

NEUBERG'S FORMS OF FERMENTATION

The fundamental equation for normal fermentation by yeast (1*st Form of Fermentation*) is :

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2.$$

Of the natural sugars it is chiefly the *d*-forms of the hexoses which ferment, namely, *d*-glucose, *d*-mannose and *d*-fructose. *d*-Galactose is fermented only by certain races of yeast. Formerly, it was assumed that the trioses were the intermediate products in fermentation but their capability for fermentation is irregular and doubtful, and only certain yeasts can ferment them. However, very recently, Meyerhof has included them, in the form of their phosphates, in the fermentation scheme (see p. 262). Pentoses and heptoses are not fermented at all. The most important step in explaining the fermentation process was taken by Neuberg who showed that in the zymase enzyme complex *carboxylase* occurs. Carboxylase is an enzyme which breaks down pyruvic acid into acetaldehyde and carbon dioxide. In this way, typical fermentation (decomposition into C_1- and C_2-compounds) is detected. The question then arises : How is pyruvic acid produced and how is acetaldehyde reduced to alcohol ? Neuberg has worked out a scheme for normal fermentation by yeast, and, although this scheme has been replaced by another (see p. 261), it will be discussed here on account of its fundamental significance.

A dehydrated glucose or its phosphate might undergo aldol depolymerisation yielding methylglyoxal and glyceraldehyde. Methylglyoxal has been detected by Neuberg[1] amongst the products of fermentation by yeast and also amongst those of glycolysis and of lactic acid fermentation. Glyceraldehyde acts first as a hydrogen acceptor and dehydrates methylglyoxal hydrate to pyruvic acid, being itself converted into glycerol. Pyruvic acid is converted (fermented) by carboxylase into acetaldehyde and carbon dioxide. It can be shown that addition of acetaldehyde stimulates fermentation by yeast. Hence, as soon as the production of acetaldehyde from pyruvic acid sets in, the former should be the chief acceptor of hydrogen from methylglyoxal hydrate (mixed Cannizzaro reaction) so that alcohol is produced. The pyruvic acid also formed should again yield acetaldehyde and carbon dioxide. The glyceraldehyde produced should either be converted into methylglyoxal by loss of water or should be transformed into dihydroxyacetone which is reconverted into hexose :

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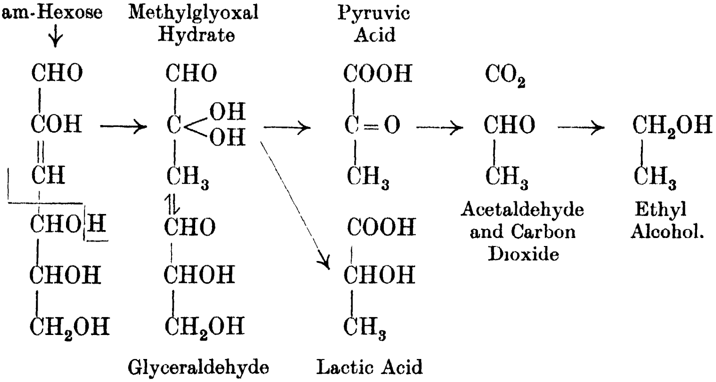
[1] **Neuberg and Kobel, *Biochem. Z.*, 1929, 207, 232.**



Methylglyoxal as such is not fermentable. Hence it is necessary to assume that labile forms of it are produced during the process of fermentation. In fermentation with yeast, lactic acid is produced in rather small proportions only. It is formed by means of an "internal" Cannizzaro reaction from methylglyoxal hydrate which is subjected to the action of *keto-aldehyde mutase.* In lactic acid fermentation and in muscle, however, lactic acid is the dominating product.

2nd Form of Fermentation :

$$C_6H_{12}O_6 = CH_3CHO + CO_2 + CH_2OH{\cdot}CHOH{\cdot}CH_2OH.$$

When bisulphite is added, the fermentation proceeds thus. The acetaldehyde produced is then at once trapped as bisulphite compound (Neuberg's process) and the glyceraldehyde produced as in the first form of fermentation acts exclusively as acceptor and is thus converted into glycerol. In this way, 48 per cent of the glucose taken is converted into glycerol. Glycerol was manufactured in this way during the war (Connstein and Lüdecke's process).

3rd Form of Fermentation :

$$2C_6H_{12}O_6 + H_2O = C_2H_5OH + CH_3COOH + 2CO_2 + 2CH_2OH{\cdot}CHOH{\cdot}CH_2OH.$$

When the reaction is alkaline the dismutation of the aldehyde is favoured so that finally acetic acid and alcohol are produced whilst, on the other hand, glycerol is produced by the hydrogenation of glyceraldehyde. Fermentation of this kind occurs in *Bacterium coli*, and to a slight extent in yeast.

A by-product of fermentation by yeast is the so-called fusel oil which consists, for the most part, of *iso*amyl alcohol and *d*-amyl alcohol. These higher alcohols are produced by the deamination and decarboxylation of amino-acids. *iso*-Leucine gives thus the optically active amyl alcohol. In addition, fatty acids such as succinic acid and formic acid are produced.

COZYMASE AND ACTIVATORS

We owe our knowledge of the specific *activator of fermentation*, the so-called cozymase, to Harden and Young. When yeast press-juice is dialysed, there remains within the membrane the thermolabile enzyme-complex of yeast, *apozymase*, a substance of high molecular weight, whilst outside the membrane there is found a thermo-stable co-enzyme, apparently of lower molecular weight, namely, *cozymase*, a *co-enzyme* indispensable for fermentation. Both substances are inactive when used separately. When they are mixed, fermentation sets in again. The co-enzyme can also be separated from press- or maceration-juice by ultra-filtration or from dried bottom-yeast by dissolution. It is not possible to separate the co-enzyme from *fresh* bottom-yeast.

Co-enzymes are dialysable substances of low molecular weight and simple chemical nature which are necessary for enzyme action. Apparently they react specifically with the enzyme-substrate compound.

In all the animal and vegetable cells investigated up to the present time cozymase has been found. Richest in cozymase are the cultivated yeasts, and indeed yeast always contains considerably more cozymase than is necessary for optimal fermentation. Frequently, half of the cozymase can be washed out of dried yeast without causing diminution in the fermentive action. Meyerhof was the first to investigate the occurrence of cozymase in animal organs. It is likewise present in blood and probably bound to the erythrocytes. The serum is free from cozymase. The green parts of plants are relatively poor in cozymase.

According to von Euler, 1 *unit of cozymase* is that amount which liberates, in 1 hour, 1 c.c. of carbon dioxide from 2 c.c. of solution containing 0·2 g. of apozymase, 0·1 g. of glucose, and 0·05 g. of phosphate (p_H 6·4). Here the apozymase is used in great excess because the rate of fermentation (evolution of CO_2) is then directly proportional to the amount of cozymase and is a measure of that amount. The degree of purity of a solid cozymase preparation is given by the ratio :

$$\frac{\text{Cozymase units}}{\text{g. of cozymase preparation}} \quad \text{or} \quad \frac{\text{c.c. of } CO_2 \text{ per hour}}{\text{g. of preparation}}.$$

Cozymase is a nucleotide, a compound composed of a purine base, a molecule of pentose and phosphoric acid. The purine base, which can be liberated by hydrolysis, is adenine. The molecular proportions of adenine, P, and reducing group are 1 : 1 : 1. The nature of the carbohydrate is not known with certainty but probably it is *d*-ribose. According to von Euler and Myrbäck,[1] cozymase consists chiefly of an adenylic acid which is identical neither with yeast nor muscle adenylic acid (see p. 84). There is produced from it, on deamination, as from animal adenylic acid, inosic acid in relatively good yield. Yeast adenylic acid has no effect at all on fermentation. Adenosine triphosphate acts as a powerful co-enzyme in animal glycolysis but muscle adenylic acid is less powerful. Thus it may be assumed that the latter is first esterified by phosphate to adenosine triphosphate. Muscle adenylic acid and adenosine triphosphate, however, cannot completely replace cozymase in fermentation by yeast.

Myrbäck's most recent investigations[2] have made it very probable that the adenylic acid in cozymase is combined with a very labile reducing residue which can easily be destroyed by heating with acid and especially by heating with alkali. From the same investigations it may be taken as certain that the cozymase from yeast is identical with that from animal organs.

Highly active cozymase may be obtained from press-juice by the method of Myrbäck and Ortenblad.[2]

Cozymase activates only fermentation by dried yeast, not that by fresh yeast. Conversely von Euler's two *activators* Z_1 and Z_2 activate fermentation by fresh yeast but not that by dried yeast. It cannot yet be said how these substances act.

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[1] See Myrbäck, *Ergebn. Enzymforsch.*, 1933, 2, 139.

[2] Myrbäck and Ortenblad, *Z. physiol. Chem.*, 1935, 233, 95, 154.

Only very recently has the significance of *magnesium* for the degradation of carbohydrates been correctly appraised. It is an indispensable activator of carbohydrate breakdown in muscle (Lohmann) and yeast (von Euler, Nilsson). It is not yet possible to say in what form, whether as a salt or in any other kind of combination, the magnesium acts. In order to remove the magnesium from the apozymase or the muscle, Lohmann uses $0{\cdot}1\ N$-KH_2PO_4. Yeast freed from Mg, that is to say, apozymase free from Mg, does not cause fermentation when mixed with magnesium-free cozymase, even in the presence of diphosphate. It can be said definitely that magnesium is necessary for the activation of phosphorylation.

Hence, fermentation occurs only when the following are present: apozymase, cozymase, magnesium, inorganic phosphate and fermentable sugar.

THE ENZYMIC SYSTEM IN FERMENTATION. THE MECHANISM OF ITS ACTION

The *zymase of yeast*, also called holozymase or panzymase, is that mixture of enzymes which must be held to be responsible for the enzyme action of yeast. The following enzyme components are included in the system:

1. **Hexases.**—Enzymes which are the first to act. They cause the formation of labile *am*-forms capable of being phosphorylated. Otherwise nothing is known about them. Meyerhof's *hexokinase* belongs to this class.

2. The **phosphatese-phosphatase system** which adds on and splits off phosphoric acid (production of zymophosphate). This system has already been discussed. It has not been decided whether this system consists of two different enzymes or of a single enzyme only which is able to shift the position of equilibrium according to variations in the concentration of the substrate.

3. **Carbohydrate redoxase**, dehydrogenase, mutase of yeast (von Euler), an oxidation-reduction system which is activated by cozymase.[1] This is the central system in the breakdown of carbohydrate by yeast (see p. 246).

4. **Glycolase**, which splits hexoses into C_3-compounds. It was detected by Neuberg in bacterial lactic acid fermentation and in fermentation by yeast.

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[1] For an account of cozymase as a vitamin see von Euler, Malmberg, Robeżnieks and Schlenk, *Naturwiss.*, 1938, 26, 45.

5. The enzyme known as **ketone-aldehyde mutase** or **methylglyoxalase** (Neuberg) which converts methylglyoxal into lactic acid.

6. **Carboxylase**, an enzyme which decarboxylates pyruvic acid (see p. 239).

7. **Carboligase**, an enzyme which forms C-C-linkages.

8. A series of **co-enzymes** and activators. In addition to those already mentioned, namely, cozymase, magnesium, and von Euler's Z_1 and Z_2 factors there are: the co-enzyme of carboxylase, namely, *co-carboxylase*[1] (Auhagen) and *co-ketone-aldehyde mutase* which has been recognised to be glutathione (Lohmann).

Of these, the only ones which have been to some extent isolated are phosphatese, carboxylase and glycolase.

The extraordinarily complicated processes which play a part in *fermentation by yeast* have by no means been explained as yet. The results of Embden's recent investigations of animal glycolysis and the isolation of *l*-glycerophosphoric acid as a product of the degradation of sugar by yeast (Nilsson) have been used also in the further study of fermentation by yeast (Meyerhof, Neuberg) as a basis for the development of altered conceptions. These are discussed later (see p. 263). The views of von Euler which, in particular, are capable of providing an explanation of the rôle of the enzymic systems will be outlined here.

At what stage in the process of fermentation does cozymase act ?

1. According to von Euler and Myrbäck the *entrance of phosphate into organic combination* and the production of carbon dioxide which, according to Harden and Young, proceeds in parallel therewith (equation I, p. 236) are proportional to the amounts of cozymase present. Hence, until the point at which production of diphosphate is reached, the presence of cozymase is necessary. As Harden and Young showed, the reaction expressed in equation II does not require the co-operation of cozymase, for isolated phosphatase transforms diphosphate into hexose.

2. Zymophosphate behaves towards unwashed, that is to say, cozymase-containing yeast and to the dehydrogenase system which it contains (*carbohydrate redoxase*, see p. 243) as an excellent hydrogen donator whilst, with washed yeast, no reducing power (decolorisation of methylene-blue) is observed. According to von Euler,

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[1] Co-carboxylase has been shown to be the diphosphate of vitamin-B_1. See Lohmann and Schuster, *Biochem. Z.*, 1937, **294**, 188. See also Ochoa, *Nature*, 1938, **141**, 831.

the reason for this is that a complementary substance, a *co-reductase* identical with cozymase, has been removed. The co-reductase and cozymase actions of cozymase preparations run parallel.

3. If dried yeast is washed it loses its power *to dismute to aldehyde* because the action of the *mutase of yeast*, as Myrbäck and Jacobi have shown, depends on the co-operation of a co-mutase which is removed by washing. This co-enzyme is likewise identical with cozymase so that one and the same co-enzyme is responsible for the phosphorylation of the zymohexoses, the reduction of methylene-blue by carbohydrate and the enzymic mutation of aldehyde.

In their work on fermentation by yeast,[1] von Euler and his school regarded as their most important aim the decision of the question whether or not the dehydrogenase which takes part in carbohydrate degradation (carbohydrate redoxase) and brings about the reduction of methylene-blue in the presence of zymophosphate is not essentially a mutase, and, as such, identical with that part of zymase which initiates the hydrolysis during fermentation, that is to say, takes part in production of diphosphate and in other reactions. Since the co-enzymes of the three processes, namely, co-reductase, co-mutase and cozymase, are identical it is all the more probable that this is actually the case. According to Wieland's view of oxidation-reduction, it is a perfectly plausible supposition that the functions of the dehydrogenase and the mutase should coincide. Further, the processes which, according to the equations III*a* and III*b* (see p. 237), lead from hexose monophosphate to diphosphate occur at the same time as fermentation of the hexose and so are connected with the oxidation-reduction processes. Thus, this third form of the action of redoxase can also be understood.

The views of von Euler[2] are summarised in the scheme shown below. According to this scheme, glucose or the hexose concerned is converted by the enzymic system which acts first, namely, the so-called *hexokinase* of Meyerhof into the *am*-form. Muscle does not contain a system of this kind. For this reason, muscle can only hydrolyse the active form of glucose spontaneously produced from glycogen : stable glucose is hydrolysed by muscle only after yeast autolysate is added. The *am*-hexose is transformed by the phosphorylating enzyme *phosphatese*, supposedly *without* the co-operation of *cozymase* (Nilsson) into monophosphate (equation III*a*), active monophosphate (Robison's ester) being the product. Two molecules of this monophosphate are decomposed, as shown in equation III*b*, by *redoxase* and *cozymase*, 1 molecule of diphosphate being produced by way of the C_3-compound, carbon dioxide and alcohol, on the one hand, and possibly by way of 2 molecules of triose, monophosphate on the other. This reaction fits perfectly into the series of mutation reactions. In the second phase of fermentation, the diphosphate is dephosphorylated *without* the co-operation *cozymase* and immediately commences the cycle of fermentation again as *am*-hexose.

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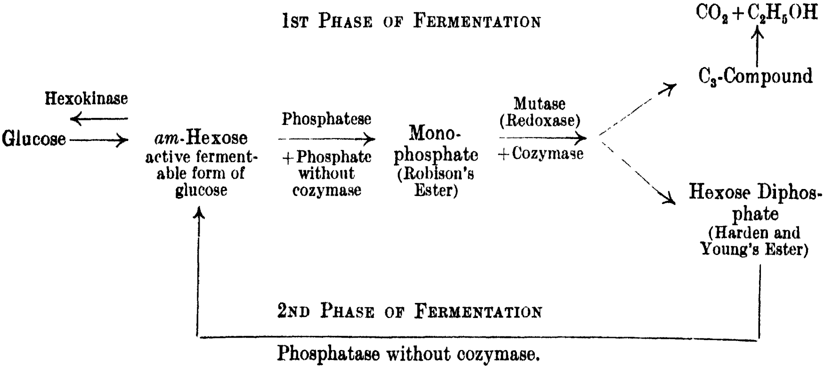
[1] *Biokatalysatoren* (Stuttgart, 1930).

[2] *Skand. Arch. Physiol.*, 1930, 59, 201.


1st Phase of Fermentation
$CO_2 + C_2H_5OH$
C_3-Compound
Hexokinase
Glucose
am-Hexose
active fermentable form of glucose
Phosphatese
+Phosphate without cozymase
Mono-phosphate (Robison's Ester)
Mutase (Redoxase)
+Cozymase
Hexose Diphosphate (Harden and Young's Ester)
2nd Phase of Fermentation
Phosphatase without cozymase.

As regards the co-operation of the enzymic system of fermentation in the *respiration of yeast with oxygen*, it should be said that it is assumed that the degradation of carbohydrate in the presence of oxygen begins with a series of reactions corresponding completely to degradation of sugar by fermentation. The C_3-compounds are, according to the customary conception, the substrates in the respiration of the hexose phosphates and this harmonises with the assumption that mutase and dehydrogenase action are due to an enzyme and its co-enzyme, namely, *carbohydrate redoxase* and *cozymase* (co-reductase) which occupy the central position in the theory of carbohydrate degradation (see p. 245).

In agreement with this is the fact that those substances, which Neuberg has said to be the intermediate products of fermentation (see below), have a remarkable effect on the respiration of yeast. Just as does sugar, they increase the respiration. The substances which act thus are: methylglyoxal, lactic acid, pyruvic acid, acetaldehyde, acetic acid and alcohol; whilst glycerol, glyceraldehyde and dihydroxyacetone have no effect.

This holds also for the dehydrogenating oxidation (respiration) of hexose phosphates by animal organs, bacteria and vegetable material in which, under certain conditions, a single hexose phosphate dehydrogenase is likewise to be regarded as the responsible agent. This agent also requires the co-operation of respiratory co-enzymes (see p. 258 ff) especially adenylic acids. Adenyl pyrophosphate, the co-enzyme of lactic acid production in muscle, as well as cozymase act as co-enzymes in the dehydrogenation of hexose phosphate by muscle extracts and extracts of the cells of plant seeds (see p. 184). On the other hand, it is not at all improbable that direct dehydrogenation (fermentation) of the sugar molecule, without preceding hydrolysis, occurs. This follows, on the one hand, from the fact that dehydrogenating enzymes exist which are adapted to act on glucose only, and, on the other hand, from the researches of Lundsgaard who showed, with muscle and yeast, that iodo-acetic acid in certain concentrations inhibits the aerobic but *not* the anaerobic phase of respiration.

The *dehydrogenating action of yeast* is advantageously studied with yeast rendered poor in reserve substances by shaking for 20 hours with oxygen.[1] Yeast can dehydrogenate *ethyl alcohol* and also *acetaldehyde* in the presence of oxygen and methylene-blue, giving acetic acid. When the concentration of acetaldehyde is high, however, it is for the most part dismuted. This again shows distinctly the relation between aerobic and anaerobic metabolism. *Lactic acid* also, and, considerably more rapidly, *methylglyoxal* are dehydrogenated by bottom yeast, *acetic acid* being produced by way of pyruvic acid. If oxygen is present, the acetic acid undergoes further dehydrogenation. In this oxidative degradation *succinic acid* and *citric acid* are obtained as intermediate products. The succinic acid is produced from 2 molecules of acetic acid each of which loses 1 atom of hydrogen from its methyl group thus:

$$2HOOC{\cdot}CH_3 - 2H = HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}COOH.$$

Since, in muscle, this process cannot be realised (see p. 158) the following is rather probably the course of reaction in the case of yeast; carbohydrate ⟶ methylglyoxal or lactic acid ⟶ pyruvic acid ⟶ acetaldehyde and carbon dioxide ⟶ acetic acid ⟶ succinic acid ⟶ fumaric acid ⟶ malic acid ⟶ oxalacetic acid ⟶ pyruvic acid and carbon dioxide. The rate of dehydrogenation of succinic acid is only about one-fifth of that of dehydrogenation of acetic acid, although the former acid must be assumed to be an intermediate product in the dehydrogenation of the latter acid. Wieland explains this phenomenon by supposing that the intermediate product is activated by the reaction which precedes its formation.

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[1] Wieland and Claren, *Annalen*, 1932, 492, 185.

Fresh yeast, because of its high content of donators, has very marked *reducing power.* Advantage is taken of this as well as of the reducing power of bacteria which stimulate fermentation in order to bring about so-called *phytochemical reductions* which have played a part, for example, in indigo vat dyeing.

YEAST PREPARATIONS

Active preparations have been prepared from yeast in a very great variety of ways. These preparations cause fermentation in the absence of the living cells.

1. **Yeast Juice** (Buchner and Hahn, Harden).—Washed pressed yeast is mixed with sand and kieselguhr and ground, and the mixture is subjected to pressure in a press. An active, dry preparation is obtained by treating the juice with ether-alcohol mixture (precipitation). Active material is also obtained by evaporating to a syrupy consistency under reduced pressure and finally drying in the incubator at 25°.

2. **Maceration-Juice** (Lebedev).—Washed yeast is so far freed from water by pressing, that it can be passed through a sieve. The sieved material is then dried for 2 days at 25°–35° (very active *dry yeast,* see below) and then ground in a coffee mill. For maceration, that is to say, complete destruction of the cellular structure, 50 g. of dried yeast are stirred with 150 c.c. of water, left for 2 hours at 35° and then filtered through a folded paper.

3. **Autolysate.**—Washed, gently pressed yeast is killed with toluene, ethyl acetate or the like, and made into a thin paste at neutral reaction. Then the destroyed cells are removed from the liquid in a centrifuge. For procedure for obtaining autolysate, see Willstätter's work on invertase.

4. **Zymin** (stable yeast dried with acetone, Buchner).—Yeast is dehydrated with acetone and ether. The material so obtained is practically free from living cells and is very active.

DETERMINATION OF THE FERMENTING POWER OF DRIED YEAST[1]

In order to determine the fermenting power of yeast press-juices and of dried yeast, various pieces of apparatus, in which the carbon dioxide produced during the fermentation is determined gravimetrically or volumetrically, are used.

1. **Gravimetric.**—Buchner determined the weight of a fermenting mixture (yeast press-juice, sugar, toluene) before, during and after fermentation, and in this way found the weight of carbon dioxide produced.

2. **Gravimetrically** by *absorbing* the carbon dioxide produced in barium hydroxide or sodium hydroxide solution.

3. **Volumetrically** by measuring the carbon dioxide evolved. For this purpose Harden and Young used a eudiometer filled with mercury and connected to the vessel in which fermentation took place. In order to prevent super-saturation with carbon dioxide, the vessel had to be shaken frequently. This can be done mechanically in the Willstätter thermostat with shaker in which serial experiments can be carried out. Here, the containing liquid is saturated solution of sodium chloride. The samples taken for fermentation should preferably be saturated with carbon dioxide. Von Euler and Myrbäck use a micro-volumetric method employing mercury as containing liquid.

4. Determination of the carbon dioxide produced by measuring the increase in *pressure* in the Barcroft-Warburg apparatus.

Experiment.—The technique of Barcroft and Warburg described for earlier experiments (see p. 186) should be applied to show, with *dried yeast*, that in fermentation with yeast the co-operation of a thermo-stable co-enzyme, *cozymase*, is necessary.

Preparation of Dried Yeast.—If a sample of dry bottom-yeast is not available use Lebedev's procedure. Obtain some fresh yeast from a brewery and wash thoroughly by decantation. Stretch a fine cloth on a frame, pour the yeast on to this, press gently, wrap in sail cloth and squeeze in a press until sufficiently dry to be capable of passing through a coarse sieve. Rub gently with the hand in order to facilitate passage through the sieve, spread out on filter paper in a layer not more than 1 cm. thick, and dry for 2 days at

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[1] Methods are given in detail in Oppenheimer and Kuhn, *Die Fermente und ihre Wirkungen*, vol. iii., pp. 1222 ff.

25°–35°. Grind the dried yeast in a coffee mill. The material thus obtained can be kept unchanged for months.

Preparation of Cozymase Solution.—In a glass centrifuge jar, carefully stir 5 g. of dry yeast to a moderately thin paste with distilled water. Then spin at high speed in a centrifuge for 10–15 minutes. Pour the yellowish supernatant liquid into a measuring cylinder. Repeat the stirring and centrifuging once or twice (not more !) In this way there is obtained a clear solution of cozymase which can be diluted with distilled water to a volume of 25 c.c. when necessary. The sediment is apozymase not yet completely free from cozymase. Mix it with water to make a volume of 50 c.c. and stir so as to produce a suspension which can be pipetted.

Take 1 g. of dry yeast in a test-tube with 8 c.c. of distilled water and boil for 5 minutes, stirring with a glass rod. Cozymase only is sufficiently stable to resist this treatment.

Samples :

	1	2	3	4	5	6
Dried yeast	0·5 g.	—	—	—	—	—
Apozymase (c.c.)	—	5	—	5	—	5
Cozymase (c.c.)	—	—	2·5	2·5	—	—
Boiled juice (c.c.)	—	—	—	—	4	4
0·067M-Phosphate buffer of p_H 6·5 (c.c.) .	1	1	1	1	1	1
0·5M-Glucose solution (c.c.) . . .	1	1	1	1	1	1
Water (c.c.)	10	5	7·5	2·5	6	1

Procedure.—Attach the vessels to the manometers and place in the thermostat at 37°. Then at once pass a current of nitrogen through each of the vessels for 5 minutes. Continue the experiment exactly as in those described on p. 191. Take readings at suitable intervals, say, every 5 to 15 minutes.

It will be found that the yeast which is to a large extent free from cozymase (sample 2) causes only slight fermentation, and that cozymase alone (3 and 5) can cause no fermentation but that apozymase and cozymase together cause extensive fermentation (4 and 6) comparable with that caused by ordinary dried yeast (1).

Fermentation does not begin spontaneously in the samples. It has been found that every fermentation process requires a certain time for incubation. Apparently, this time is required because the sugar, before it can be fermented, must be phosphorylated and the phosphorylation takes some time. It is therefore advisable to add, at the start, a quite small amount of hexose diphosphate in order to reduce this period of incubation. The calcium salt (" candiolin ") of hexose diphosphate is preferably used because it is easily obtained.

Experiment.—Measure the *anaerobic fermentation and the respiration of fresh bakers' yeast,* using duplicate samples very similar to sample 1 in the preceding experiment. In each vessel take 0·2 g. of fresh yeast, preferably in the form of a suspension. In this experiment also fill the vessels with nitrogen before measuring the extent of fermentation. Determine the respiration in vessels containing air and also a small receptacle containing potassium hydroxide solution (5 per cent) to absorb the carbon dioxide. Since in both cases the partial pressure of only one gas changes the " constants " of the vessels, K_{CO_2} and K_{O_2} can be used in the calculation.

THE PASTEUR-MEYERHOF EFFECT

Yeast has an aerobic and an anaerobic metabolism (*fermentation* and *respiration*). Pasteur's theory seeks to make the biological significance of these processes and the relation between them to some extent, at least, comprehensible. It states that fermentation is an intramolecular respiration (*vie sans air*) and serves as a substitute for respiration with oxygen as a means of meeting the energy requirements of the vital processes. It was held for long that this theory was refuted.

Meyerhof showed that *muscle* glycogen is first degraded anaerobically to lactic acid (glycolysis) which, in the aerobic phase, is partly reconverted into glycogen. The energy necessary for these processes is obtained by the oxidation, to carbon dioxide, of glucose or of an intermediate product not yet exactly characterised. The ratio of carbon dioxide produced to oxygen consumed $CO_2 : O_2$, the so-called *respiratory quotient* is equal to 1. In the total balance it is found that one equivalent of lactic acid is oxidised for 3–6 molecules reconverted into glycogen (Meyerhof's reaction).

According to Meyerhof and Warburg, however, the anaerobic and aerobic metabolisms of *tumours*, *yeasts* and *lactic acid producers* are connected with each other in the same way so that in the Meyerof reaction we deal with a general biological principle. In carbohydrate metabolism, then, there is only a *single* fundamental mechanism which is common to all cells and exhibits quantitative differences only. Briefly, *anaerobic* metabolism leads, in all living cells, to the production of C_3-compounds, such as lactic acid, methylglyoxal and so on. In the *aerobic* phase, when oxygen is present, the products of the breakdown are completely (lactic acid in normal muscle) or only partly (as in yeast, methylglyoxal in yeasts, lactic acid in bacteria and tumours) reconverted into carbohydrate. The ratio of the amount which disappears (or in other words, the amount not converted into C_3-compounds) to the amount oxidised is roughly constant in all cases.

With *yeasts* the position is as follows: A distinction must be made between top and bottom yeasts. The former collect at the surface; the latter, at the bottom of the vat, in which the fermentation takes place. The respiration of the bottom yeasts is only very slight as a result of long continued adaptation. With them, oxygen has no appreciable effect on the respiration: the Pasteur-Meyerhof principle can scarcely be observed to hold for them. In all cases oxygen is an important factor in the propagation and reproduction of yeast, but this has nothing to do with the problem under discussion. Top yeasts, [pressed yeast, bakers' and wild yeasts (*Torula*)] consume very considerable amounts of oxygen. From what has been said above, it would be expected, on the basis of the Pasteur theory, that in these cases anaerobic fermentation would be inhibited or prevented by admission of air because the C_3-compounds or the alcohol and the carbon dioxide produced from them by fermentation could not be produced. Meyerhof showed that this is so. With a series of races of yeast he found that, in fermentation with yeast as in all other cases, for each part of hexose oxidised (in the aerobic phase), six to twelve parts of intermediate product are produced (or an equivalent amount of sugar is not fermented). In other words:

$$\frac{\text{Amount of material reconverted into carbohydrate (molecules of sugar)}}{\text{Amount of material oxidised (molecules of sugar)}} = 3-6.$$

This quotient can be determined by measuring the total amount (in fermentation and respiration) of carbon dioxide produced aerobically and also the amount of oxygen taken up. Since the respiratory quotient is 1 this amount of oxygen corresponds to the carbon dioxide of respiration. The difference is the carbon dioxide of aerobic fermentation. The amount of carbon dioxide produced anaerobically is also measured. From these data it may easily be calculated how many molecules of sugar are not broken down (or are replaced by resynthesis) when 1 molecule of sugar is oxidised, *i.e.* is broken down during respiration. According to Warburg, the actual Meyerhof quotient in lactic acid production is :

$$\frac{\text{Molecules of lactic acid which disappear}}{\text{Molecules of } O_2 \text{ used up}}.$$

The corresponding expression for fermentation is :

$$\frac{\text{Molecules of } CO_2 \text{ of fermentation which disappear (are not produced)}}{\text{Molecules of } O_2 \text{ used up}}.$$

It can easily be seen that the quotient calculated from the sugar must be three times larger, for, from 1 molecule of sugar there are produced, in fermentation, 2 CO_2 (numerator) and in respiration 6 CO_2 (denominator).

Another important point is that the whole cycle of reactions included in the Pasteur-Meyerhof effect, that is to say, in respiration and fermentation, is a vital function and hence depends on the maintenance of the structure of the yeast. Oxygen, it is true, inhibits fermentation by yeast dried with acetone, and fermentation by Lebedev's maceration-juice also, but it is supposed that this is due to oxidation of a catalyst (see below).

Lipman, using Meyerhof's muscle extract, investigated the effect on glycolysis of oxidising agents, such as iodine or quinone, as a substitute for oxygen in order to determine whether the Pasteur effect could be regarded as an *oxidative inhibition of glycolysis.* In muscle extracts, glycolysis is independent of the presence of oxygen since the respiration system is absent but is inhibited, on the other hand, by quinone and iodine. According to these results the enzyme of glycolysis appears to be capable of *reversible* oxidation. In the cells, the enzyme which produces lactic acid is believed to be inactivated in the presence of oxygen by the auto-oxidisable respiratory enzyme, so that glycolysis is prevented. If so, this effect is then the so-called Pasteur-Meyerhof effect.

ANIMAL GLYCOLYSIS

THE MECHANISM OF GLYCOLYSIS[1]

In addition to anaerobic fermentation, the anaerobic degradation of carbohydrates in the animal cell is one of the glycolytic processes.

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[1] Meyerhof, *Die chemischen Vorgänge im Muskel* (Berlin, 1930).

As early as the year 1847, Liebig observed that sugar is converted into lactic acid by animal tissues. The physiological function of glycolysis is the provision of the small amounts of energy required for the synthetic processes of the cells, these amounts being set free as a result of the conversion of glucose into its degradation products (lactic acid, alcohol and carbon dioxide, and so on). The amount of energy thus produced is small : it is only about 5 per cent of the amount produced in the combustion of glucose. However, as a result of the large amount of material transformed, considerable amounts of energy are liberated. The whole mechanism serves as a reserve of energy. *Respiration with oxygen* depends, of course, on the rate of diffusion of the oxygen in the cells and this rate also limits the amount of energy produced by the degradation of glucose. Accordingly, when *great amounts of energy* are required in a short time, as, for example, during *muscular contraction, anaerobic glycolysis* sets in (1). The fact that when sufficient oxygen is supplied to normal tissue no glycolysis at all occurs but that glycolysis does occur when oxygen is absent, is in agreement with what has been said above. This replacement of glycolysis by respiration illustrates the so-called Pasteur-Meyerhof effect (see p. 251).

It is not known what causes muscular contraction. At present also, it cannot be stated with certainty what phases occur in the course of glycolysis. Every chemical investigation of the intermediate stages of processes of this kind is, of necessity, dependent on disturbance of the co-ordination of the cell in consequence of inhibition phenomena, destruction of the cell, and so on, and hence processes may occur which do not take place in the intact normal cell. The mechanism of the chemical processes which occur during and after work has been found, in recent years, to be much more complicated than was formerly supposed. It can at least be taken as certain, that the anaerobic degradation of carbohydrate in animal tissues leads to the production of lactic acid [*l*(+)-lactic acid, sarcolactic acid][1] which appears as a result of stabilisation and is reconverted into carbohydrate in muscle (Pasteur-Meyerhof effect). More is known about the amounts of energy liberated during muscular contraction. Half of this amount can be obtained in the form of productive work. Fundamentally, the *reaction which supplies energy* is the *oxidation of carbohydrate by oxygen* (2) which commences at once when contraction begins, proceeds slowly and also continues for a long time after the work is finished. This explains the increased respiratory and cardiac activity after bodily exertion. *Lactic acid production* (1), on the other hand, is a process which occurs relatively quickly and is complete a few seconds after the work is finished.

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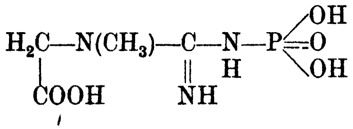
[1] For the preparation of this acid, see *J. Biol. Chem.*, 1933, 99, 155.

In 1927 an acid-labile phosphate known as *phosphagen* was discovered in striped muscle from the frog by P. and G. P. Eggleton. Phosphagen decomposes during muscular activity. Fiske and Subarow showed that the phosphagen of the muscle of warm-blooded animals is an easily hydrolysed *creatine phosphate.* Some time later, Lundsgaard succeeded in preventing glycolysis, formerly held to be the specific reaction primarily responsible for the contraction, without interfering with the capability of the muscle to react to stimulation. He achieved this by adding *mono-iodo-acetate.* It has been found that iodo-acetic acid, like fluoride, inhibits phosphorylation. This led to the discovery of the *third process in muscle which supplies energy*, namely, the degradation of *phosphagen* (3). Phosphagen (formula *A*), a compound, as has been said, of creatine (which occurs, in large amounts, in animal tissues) and phosphoric acid, provides, on hydrolysis, an amount of energy which alone, without glycolysis, suffices to meet the energy requirement (12,000 cal.) of muscular work for a short time.



$$\underset{\substack{|\\ \mathrm{COOH}}}{\mathrm{H_2C}}-\mathrm{N(CH_3)}-\underset{\substack{\|\\ \mathrm{NH}}}{\mathrm{C}}-\underset{\mathrm{H}}{\mathrm{N}}-\mathrm{P}\begin{smallmatrix}/\mathrm{OH}\\ =\mathrm{O}\\ \backslash\mathrm{OH}\end{smallmatrix} \qquad . \quad . \quad . \quad . \quad . \quad A$$

Degradation of phosphagen occurs only during work; it is probably the *primary* process for the supply of energy. The *relation* between the energy-supplying processes is believed to be as follows: When the muscle is stimulated phosphagen is degraded and contraction takes place. The glycolytic production of lactic acid follows more slowly. The energy set free in this last process is used to re-synthesise phosphagen from creatine and phosphoric acid. Muscle can continue to work for a long time when glycolysis continues to provide for the re-synthesis of phosphagen. During the phase of recovery, carbohydrate or lactic acid is oxidised by oxygen (Meyerhof). The energy of this process serves to reconvert lactic acid into glycogen as has been found to be the case in muscle and liver (Pasteur-Meyerhof effect).

Glycolysis is inhibited by *mono-iodo-acetic acid*, re-synthesis of phosphagen does not occur and the muscle becomes exhausted. Under anaerobic conditions, lactic acid accumulates until a maximal amount is present and the muscle can no longer work. It is different when oxygen is present. Then, the lactic acid is oxidised and the muscle remains able to work.

Glycolysis is particularly vigorous in *cancer cells* and also in the grey matter of the brain, in the retina and in embryonic tissue. The *respiration of cancer cells*, however, is much smaller than that of embryonic tissue. The Pasteur-Meyerhof effect is not observed in cancer cells. In them, glycolysis continues as before in presence of oxygen. Aerobic glycolysis may be produced in normal embryonic tissue by inhibiting respiration with hydrocyanic acid or by checking the Pasteur-Meyerhof effect with propionitrile, glutathione, or in some other way. In contrast to that of cancer cells, the vital power of such normal tissue is limited, however.

Warburg, who has studied the question in detail, has proposed a theory of the *formation of cancer cells* in the organism. The only cells which need be considered in connexion with the total glycolysis of the tissue are undifferentiated cells of embryonic character which cause vigorous glycolysis, since other cells cannot degrade sugar anaerobically. When there is a lack of oxygen resulting from damage to the cells, caused by crushing, for example, or else by injury to the oxidative enzyme system, only those cells which can no longer cause glycolysis will die. The embryonic glycolysing cells, however, can continue to live and grow into genuine cancer tissue, which has only a feeble respiration. In 1926 Charrel showed that embryonic tissue, in which the respiration was reduced so far by addition of arsenic that its metabolism was at the same level as that of cancer cells, develops into fatal cancerous tumour after being transplanted to the animal body.

THE LACTIC ACID ENZYME

The *substrate of glycolysis* in muscle is *glycogen*. In intact muscle and also in muscle pulp the study of glycolytic action encounters obstacles because of the high glycogen content. Meyerhof prepared an *extract* which produces lactic acid from glycogen and starch by extracting cooled muscle with isotonic solution of potassium chloride or distilled water. He also succeeded in separating from erythrocytes the enzyme which produces lactic acid. A scheme very similar to that drawn up for fermentation by yeast has been drawn up for animal glycolysis. According to Meyerhof the first stages of phosphorylation by the *phosphatese-phosphatase system* are in the main the same in both cases (see p. 236). Fermentable hexoses, such as glucose, fructose or mannose, are not always broken down by extracts of fresh muscle. Apparently, muscle extract, in contrast to yeast, lacks an enzyme capable of preparing hexoses for the glycolysis. Meyerhof isolated this preparing activator from yeast in the form of the so-called *hexokinase* (see p. 245). To it he attributes the power to produce the *am*-hexoses necessary for phosphorylation. It is assumed that, when glycogen undergoes glycolysis, these *am*-hexoses are produced directly.

Hexokinase is a water-soluble substance having the properties of an enzyme and sensitive to heat. According to von Euler, a similar substance occurs also in the muscle of warm-blooded animals. But it is not certain that this substance is identical with hexokinase.

Neuberg succeeded in showing the production of *methylglyoxal*, both during glycolysis in muscle and in alcoholic fermentation.[1] Under certain conditions (low concentration of enzyme and substrate) he was able, by long autolysis at 37° in presence of toluene, to destroy that part of the enzyme necessary for the further transformation of the keto-aldehyde. With hexose diphosphate as substrate—when the dismuting part of the enzyme is destroyed, the phosphorylating power is also lost—methylglyoxal accumulates and can be trapped as osazone with dinitrophenylhydrazine.[2] The yield of methylglyoxal is just short of 100 per cent of the theoretical calculated on the amount of diphosphate transformed. Whilst with yeast (see p. 239), methylglyoxal is converted into pyruvic acid or acetaldehyde and carbon dioxide or glycerol or alcohol; when lactic acid is produced, it is produced from methylglyoxal by addition of water as a result of the action of *methylglyoxalase*.

In addition to that form of glycolysis which is preceded by phosphorylation, however, there is certainly another route by which carbohydrate is degraded *without* preceding *phosphorylation*.[3] According to Kraut and Bumm, a co-enzyme *T* is a characteristic constituent of all tissues which cause glycolysis without phosphorylation. *T* is possibly identical with pyruvic acid.

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[1] *Biochem. Z.*, 1929, 207, 232.

[2] For the preparation of this substance, see *J. pr. Chem.*, 1894, 158, 258.

[3] According to Holms and to Bumm, sliced brain, retina and embryonic tissue cause glycolysis of glucose even in the absence of phosphoric acid.

The *enzyme systems* also show far-reaching resemblances. Just as zymase can be shown to consist of the thermo-labile apozymase and the thermo-stable cozymase so also does the enzyme system of glycolysis consist of two such parts, the actual enzyme and its co-enzyme (Meyerhof).

According to von Euler, here also a *carbohydrate redoxase* occupies the central position in the process.

The *co-enzyme in lactic acid production*, which, like cozymase in fermentation by yeast, is indispensable in glycolysis in muscle, is, according to Lohmann, *adenosine triphosphate* in which two phosphate groups are loosely bound in the form of a pyrophosphate residue. Consequently, it is not identical with cozymase which, indeed, also occurs in muscle and is isomeric with adenyl monophosphate (*muscle adenylic acid*) which may be produced by elimination of pyrophosphoric acid from the triphosphate. The production of lactic acid in muscle, however, can be accelerated by similar systems from yeast (see p. 242). According to Holmberg, adenosine triphosphate acts by transferring inorganic phosphate to the sugar, thereby rendering it capable of yielding lactic acid.

I. Glucose + Adenosine triphosphate $\xrightarrow{\text{Phosphatase}}$ Hexose diphosphate + Adenosine monophosphate.

II. Adenosine monophosphate + $2H_3PO_4$ $\longrightarrow$ Adenosine triphosphate.

In any case, the co-enzyme co-operates in the intermediate phosphorylation and dephosphorylation and the higher the degree of phosphorylation of the substrate the smaller is the amount of co-enzyme required. Hence, the glycolysis of glucose requires more co-enzyme than does that of mono- or di-phosphate. In addition to adenyl pyrophosphate glycolysis, like fermentation by yeast, requires *inorganic phosphate* and *magnesium*. If a muscle extract lacks one of these constituents, esterification of the sugar and production of lactic acid from glycogen do not occur. No place of special importance can be assigned to any constituent of the whole enzyme system—neither to the co-enzyme nor to the magnesium. It cannot be said, with certainty, exactly at what point in the production of lactic acid the co-enzyme system acts.

According to Lohmann, the *co-enzyme for the degradation of creatine phosphoric acid* is likewise adenyl pyrophosphate. The production and also, indirectly, the decomposition of this pyrophosphate are coupled with the breakdown of creatine phosphoric acid into creatine and phosphoric acid.

$$\text{Adenyl pyrophosphate} \longrightarrow \text{Adenylic acid} + 2H_3PO_4.$$
$$\text{Adenylic acid} + 2\ \text{Creatine phosphoric acid} \longrightarrow \text{Adenyl pyrophosphate} + 2\ \text{Creatine}.$$

The aerobic degradation of carbohydrates in animal tissue, that is to say, *respiration*, is probably a direct continuation of the anaerobic process. It is known that lactate is easily broken down by respiration whilst, on the other hand, many carbohydrates are broken down by respiration without preceding glycolysis. Since the presence of iodo-acetic acid inhibits the glycolytic process, this acid serves to decide which route is preferred for oxidation. In yeast poisoned with iodo-acetic acid, respiration proceeds almost unchanged (Lundsgaard), but, on the other hand, in brain, testicle and sarcoma poisoned in the same way, it is inhibited; the inhibition being counteracted by lactate (Krebs). It follows that in the first case it is the glucose, as such, which is degraded; but in the second case the lactic acid. As to the mechanisms of oxidation involved, see p. 158. The *co-enzyme of the main process of respiration* in which lactic acid is involved, is not identical with adenyl pyrophosphate or cozymase, however, and so is different from the co-enzyme of anaerobic degradation of sugar (Szent-Györgyi). It is supposed to be a flavin compound (see p. 228) called by Szent-Györgyi[1] *cytoflav*. Cytoflav affects that part of the respiration sensitive to the action of arsenic, whilst arsenic does not affect the increase of respiration produced by adenyl pyrophosphate and hexose diphosphate.

As to the *co-enzyme systems* mentioned, see p. 84.

EMBDEN'S SCHEME

A number of very recent discoveries, especially those made during the study of glycolysis, indicate that *methylglyoxal* is probably not an intermediate product. Since the investigations on which this new conception is based are not yet complete, the older views on the fermentation by yeast have been discussed (see p. 240) not least because it seemed necessary to do so on historical grounds as well.

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[1] *Biochem. Z.*, **1932**, **246**, **203**.

Neuberg himself has always pointed out that synthetic methylglyoxal, which, of course, is not fermentable, should be regarded only as a stabilised form of a labile methylglyoxal which can be held to be the actual intermediate product. In the case of yeast, the keto-aldehyde is produced by the enzyme *glycolase* without the co-operation of a co-enzyme. At the same time, some lactic acid is always produced because of the action of keto-aldehyde mutase, the co-enzyme of which, according to Lohmann, is glutathione. It can be shown that, by the addition of glutathione, the production of lactic acid by yeast is considerably increased. It must also be borne in mind that both glyceraldehyde and dihydroxyacetone yield the same osazone as does methylglyoxal (Wohl), so that it is very possible that the methylglyoxal is produced during the process of isolation and is not a degradation product at all.

Just as the inhibition of glycolysis by iodo-acetic acid led to the discovery of phosphagen metabolism, so did the recent discovery by Embden, that another part of the glycolytic process is inhibited by *fluoride*—probably inhibition of dephosphorylation—lead to the isolation of an intermediate product which suggests a mechanism of sugar degradation different from that usually assumed up to the present.

Embden, Deuticke and Kraft[1] obtained the ester of l-*glycerophosphoric acid* from glycogen degraded by *muscle poisoned with fluoride* and isolated this ester as barium salt. The ester was obtained in still larger amounts from hexose diphosphate. In addition, this hexose phosphate, when treated with muscle, yields *glyceryl monophosphate* which can likewise be isolated as barium salt.[2] These discoveries necessarily lead to the assumption that the phosphoric acid is eliminated not during but after the breakdown of the C_6-chain (see p. 257)—an assumption which contradicts that previously made.

Thus, according to Embden, the degradation of hexose diphosphate should be formulated as follows (I–III):

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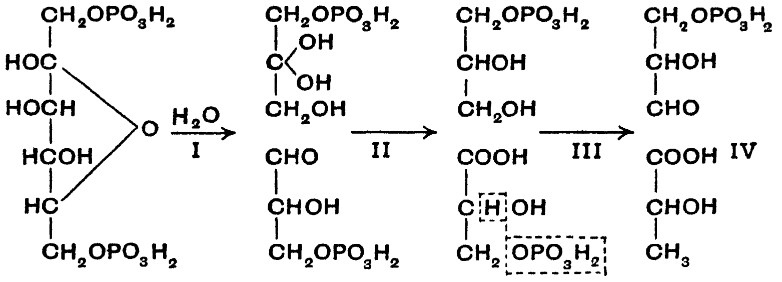
[1] *Klin. Woch.*, 1933, 12, 213.

[2] Meyerhof and Kiessling, *Biochem. Z.*, 1933, 264, 40.



The diphosphate (I) decomposes first into 2 molecules of a triose monophosphate (II) which take up water and undergo dismutation to the phosphoric esters of glycerol and glyceric acid (III). These two phosphoric esters are further transformed by *non-poisoned* muscle pulp ; hence their significance as intermediate products in glycolysis. Phosphoglyceric acid *alone* very readily yields *pyruvic acid* (III), phosphoric acid and some *lactic acid.* Of this pyruvic acid, 80 per cent can be isolated. Moreover, with hexose diphosphate as starting material, the production of pyruvic acid by muscle extract can also be directly detected by trapping with sulphite. In this case also, α-glycerophosphoric acid is produced as dismutation product (Meyerhof and McEachern). If glycerophosphate is also present at the same time in addition to phosphoglyceric acid an extraordinarily great *increase in the amount of lactic acid produced* is observed, more lactic acid being obtained than corresponds to the quantity of phosphoglyceric acid present. According to Embden, Deuticke and Kraft, pyruvic acid in presence of muscle pulp undergoes hydrogenation to lactic acid, the glycerophosphoric acid being converted into triose phosphate (IV) which is again dismuted, and so on. The laevo-form only of glycerophosphoric acid reacts in this way. Meyerhof and Kiessling have confirmed these findings. The above-mentioned dismutation of d-*glyceraldehyde phosphoric acid* (triose phosphate) in muscle has also been experimentally confirmed by Meyerhof. The phosphoglyceric acid thereby formed again causes production of pyruvic acid which, according to Hahn, always occurs in muscle. The pyruvic acid is converted into lactic acid, and so on, until, finally, both the phosphoric esters are transformed into lactic acid.

Finally, Meyerhof and Kiessling (*loc. cit.*), by allowing hexose diphosphate to act on dialysed muscle extract, isolated the triose-phosphoric acid (I–II) in large yield, in the form of *dihydroxyacetone* *phosphoric acid.* This cleavage of the carbon chain is brought about by the enzyme *zymohexase* which establishes an equilibrium greatly dependent on temperature and very rapidly attained from both sides.[1]

Lactic acid production from glycogen is inhibited by considerably smaller amounts of *fluoride* than is the production from hexose diphosphate. For 50 per cent inhibition, $0{\cdot}001M$-fluoride suffices in the first case, whilst in the second $0{\cdot}01M$ is required. According to Meyerhof and Kiessling, fluoride inhibits not the conversion of the diphosphate into the two monophosphates, but the breakdown of phosphoglyceric acid into pyruvic acid and phosphoric acid. On the other hand, according to the same authors, *iodo-acetic acid* inhibits the conversion of pyruvic acid plus glycerophosphoric acid into lactic acid, complete inhibition being caused by $0{\cdot}001M$-iodo-acetic acid.

The chief difference between the Embden and the Neuberg schemes is that, although both begin at hexose diphosphate, in Embden's scheme not methylglyoxal but pyruvic acid forms the central point in the process.

Although Neuberg's scheme has proved very fruitful in explaining the glycolytic process, Embden's scheme, by comparison, presents advantages. The rôle of phosphoric acid in glycolysis and fermentation is clearly explained by the assumption of the breakdown of the diphosphate into 2 triose phosphates. Methylglyoxal is converted by muscle into *d*(–)- and *l*(+)-lactic acid, whereas in metabolism only *l*(+)-lactic acid is encountered. As has already been pointed out, the assumption that another form of methylglyoxal takes part in metabolism can only be regarded as an expedient. The lactic acid produced from pyruvic acid and glycerophosphate, on the other hand, consists exclusively of the physiological sarco-lactic acid. This fact strongly supports the new scheme. Finally, the co-operation of methylglyoxalase in glycolysis is rather improbable because this enzyme requires, as co-enzyme, reduced glutathione which, however, is *not* necessary for glycolysis (Lohmann). According to Meyerhof, the production of methylglyoxal observed in extracts containing the enzymes of muscle and yeast results from the fact that the exceedingly labile triose phosphoric acid, because the enzyme system is damaged, is not dismuted in the normal way but breaks down, purely chemically, into methylglyoxal and phosphoric acid.

It is natural to apply the scheme for the production of lactic acid to *alcoholic fermentation*, especially since pyruvic acid (and acetaldehyde) and glycerol (in the presence of bisulphite) are encountered during this process. As early as 1930, Nilsson obtained *phosphoglyceric acid* from hexose diphosphate by the action of yeast poisoned with fluoride and this discovery led to the institution of similar investigations on muscle. Lohmann found that a difficultly hydrolysed phosphoric ester is produced from hexose diphosphate by the action of yeast maceration-juice poisoned with fluoride, and Meyerhof proved that this ester consists of *glycerophosphoric acid* and *phosphoglyceric acid* and that, in this case also, dismutation occurs. It has not yet been definitely proved that *glyceraldehyde phosphoric acid* can be dismuted by enzyme free from phosphatase but it has been shown, however, that this acid undergoes the same transformation with sodium fluoride and maceration-juice as in muscle extract. According to Smythe and Gerischer, dextrorotary glyceraldehyde phosphoric acid is converted by fluoride and maceration-juice into *l*-*a*-glycerophosphoric acid.

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[1] Meyerhof and Lohmann, *Biochem. Z.*, 1934, 271, 101.

Neuberg and Kobel have shown that both synthetic dl-*phosphoglyceric acid* and the natural *l*-form are converted into *pyruvic acid* by yeast, lactic acid bacteria and a bacterium closely related to these last, namely, *Termobacterium mobile.* At the same time, under certain experimental conditions, decarboxylation occurs with production of *acetaldehyde* and *carbon dioxide.* If the hydrogen-ion concentration is suitable, and not fresh yeast but yeast preparations, such as dried yeast, maceration-juice and yeast dried with acetone, are used, this becomes the main reaction. This decarboxylation can readily be understood since there occurs in yeast, as is well known, the enzyme, *carboxylase,* not found in muscle. Nilsson's only assumption which has not yet been experimentally grounded is that the oxidation-reduction system acetaldehyde-triose phosphoric acid is made to explain the occurrence of the first form of fermentation (production of alcohol), whilst in the case of glycolysis, on the other hand, the oxidation-reduction system pyruvic acid-glycerophosphoric acid is made responsible for the production of lactic acid.

Also, according to Meyerhof, the acetaldehyde produced during fermentation by yeast does not react with glycerophosphoric acid, but with glyceraldehyde phosphoric acid—alcohol and phosphoglyceric acid being produced. The latter reacts in the usual way.[1]

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[1] For Meyerhof and Kiessling's most recent views on the mechanism of fermentation, see *Biochem. Z.*, 1933, **267**, 313; *Ergebn. Enzymforsch.*, 1935, **4**, 220.

DETERMINATION OF LACTIC ACID PRODUCTION IN MUSCLE EXTRACTS[1]

Isolation of the Lactic Acid-producing Enzyme.—High concentrations of the enzyme are obtained both from muscle itself and from the carbohydrates present in muscle by cooling in ice, crushing in water and then centrifuging the extract. Isotonic potassium chloride solution or distilled water is used. The experiments described below are carried out with an extract obtained thus. Dry preparations of the enzyme cannot be obtained from the extract. The co-enzyme system (inorganic phosphate, adenyl pyrophosphate and magnesium) can be removed by dialysis, the residual extract being then inactive towards glycogen and to phosphoric esters of the carbohydrates (Lohmann). If suitable additions are made to the extract, various parts of the glycolytic process can be isolated (see above). Considerable glycolysis can be brought about by the extract in fermentable hexoses if hexokinase from yeast is added (see above). The lactic acid produced may be determined manometrically (by Warburg's method) or chemically. The chemical method is chosen here, the manometric procedure being described in the study of bacterial lactic acid fermentation.

If frog muscle is used for the extraction of the enzyme, only frogs of the species *Rana temporaria* are suitable. Do not cut up the muscle. Freeze it slightly at $-1°$ to $-2°$ and crush in potassium chloride solution or in distilled water. Do not allow the temperature to rise above 0° because, if this happens, the enzyme solution becomes more unstable. (The extract obtained in this way is almost free from carbohydrate, the efficiency of extraction being nearly equal with potassium chloride solution and distilled water; however, the activity of the distilled water extract is frequently higher than that of the other, and failures, which are due to insufficient cooling, are rarer.) When extracting with potassium chloride solution, use four parts of it to three parts of muscle and when extracting with distilled water take three parts of it to two of muscle. The extracts can be kept in a state suitable for use in glycolysis experiments for half a day in a freezing mixture at $-3°$ although their activity continuously decreases. An extract having fairly good keeping quality is obtained from rabbit muscle.

Experiment.—The experiment is carried out at a temperature of 20°, or else at room temperature. Since the Pasteur-Meyerhof effect does not occur with muscle extracts and since, accordingly, oxygen does not interfere with the glycolysis (see p. 252) it is possible to work under aerobic conditions.

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[1] Meyerhof, *Biochem. Z.*, 1926, 178, 395.

The following are to be determined :

(1) The amount of lactic acid spontaneously produced without addition of polysaccharide.

(2) The amount produced in the presence of glycogen.

(3) The amount produced from hexose diphosphate (Harden and Young's ester).

(4) The amount produced in the presence of starch.

Take four conical flasks (1, 2, 3 and 4 ; capacity 25 c.c.) and into each pour 5 c.c. of frog-muscle extract freshly prepared with distilled water (corresponding to 2 g. of muscle for each flask) and 1 c.c. of $0{\cdot}4M$-sodium bicarbonate solution. Into flask No. 2, pour 1 c.c. of 4 per cent glycogen solution (Merck) ; into flask No. 3, 1 c.c. of $0{\cdot}4M$-sodium hexose disphosphate [1] ; and into No. 4, 1 c.c. of $0{\cdot}4$ M-starch. Make up to a total volume of 10 c.c. in each case with distilled water. Do not add phosphate. Duration of the experiment : 3 hours.

Determination of the Lactic Acid Produced.—For the determination of the lactic acid, it is preferable to use the procedure of Fürth and Charnass according to which the acid is oxidised to acetaldehyde with a boiling, feebly acid solution of potassium permanganate. The acetaldehyde is distilled, as soon as it is produced, into a solution of potassium bisulphite and is there determined iodometrically. Substances which yield products capable of combining with bisulphite, carbohydrates and proteins, for example, must first be removed. In the present case, the mixture is deproteinised with 10 per cent sodium tungstate solution and $0{\cdot}67\,N$-sulphuric acid, according to the procedure of Folin and Wu, and the carbohydrates are removed with copper sulphate and calcium hydroxide according to the method of van Slyke and Clausen.

Procedure.—(a) *Removal of protein and sugar.*[2]

Solutions required :

(1) Sulphuric acid, $0{\cdot}67\,N$.

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[1] For the preparation of this compound, see Oppenheimer and Kuhn, *Die Fermente und ihre Wirkungen*, vol. iii., pp. 406 ff.

[2] According to Lohmann in Oppenheimer and Kuhn, *op. cit.*, vol. iii., pp. 1264 ff.

(2) Sodium tungstate solution, 100 g. in distilled water to make 1 litre of solution.

(3) Copper sulphate solution ($CuSO_4 . 5H_2O$), 100 g. in distilled water to make 1 litre of solution.

(4) Calcium oxide suspension, 100 g. of analytically pure CaO suspended in distilled water to make 1 litre of suspension.

Quench the calcium oxide with 400 c.c. of distilled water in a porcelain basin and make up to 1 litre. Shake up each time before removing the desired amount with a pipette.

Pour the contents of the four conical flasks into four others of 100 c.c. capacity each, add to each from a pipette 10 c.c. of 10 per cent sodium tungstate solution, and shake up. Then pour into each flask 10 c.c. of 0·67 N-sulphuric acid, shaking vigorously meanwhile. Close the flasks with rubber stoppers and shake vigorously again. Remove the precipitates quantitatively by filtration (they are easily filtered). Do not wash the material removed. Of each of the filtrates, take 25 c.c. in four clean 100 c.c. conical flasks and to each flask add 10 c.c. of the suspension containing calcium hydroxide and 6 c.c. of the copper sulphate solution. After ½-hour when the colour of the repeatedly shaken mixtures has changed from greenish to blue, filter at once, and of the filtrates (which must always be alkaline and at most faintly blue in colour) take aliquot portions (10 c.c.) for the lactic acid determination.

(b) *Determination of lactic acid.*

Solutions required :

(1) Sulphuric acid, 5 per cent solution.

(2) Potassium permanganate solution, 0·1 N. (From this solution prepare the 0·002 N-solution by dilution.)

(3) Potassium bisulphite solution (metabisulphite, Kahlbaum).

(4) Iodine solution, 0·1 N. (From this solution prepare 0·01 N-solution by dilution.)

(5) Starch solution, 1 per cent in saturated solution of potassium chloride.

The potassium permanganate solution need not be standardised but the titre of the iodine solution must be known exactly.

The bisulphite solution must not be more than 2 or 3 weeks old. Keep it in a brown bottle. To obtain the starch solution, boil 1 g. of soluble starch suspended in 10 c.c. of water with 90 c.c. of saturated solution of potassium chloride. The water used must be *doubly* distilled. To prepare it, distil ordinary distilled water from a flask of Jena glass containing barium hydroxide.

The prepared liquid in a Kjeldahl flask (capacity 100 c.c.) is neutralised with sulphuric acid, acidified with 1·5 c.c. of 5 per cent sulphuric acid, made up to a volume of 30 c.c., and boiled. The lactic acid is oxidised to acetaldehyde in the boiling liquid with 0·002 *N*-permanganate solution, added drop by drop. The distilled acetaldehyde is trapped in potassium bisulphite solution which is then titrated with 0·01 *N*-iodine solution, using starch as indicator. Sodium bicarbonate is then added to liberate the bound acetaldehyde and the titration is continued to the end. The method is suitable for the determination of 0·1–2·0 mg. of lactic acid. 1 mg. of the acid is equivalent to 2·22 c.c. of 0·01 *N*-iodine solution.

Apparatus (see Fig. 33).—The apparatus consists of a Kjeldahl flask of pyrex glass provided with a two-holed rubber stopper. Through one hole is passed the capillary stem of a small dropping funnel: through the other, a doubly bent glass tube connected to a Liebig condenser about 25 cm. long. The tube of the condenser (diameter 8 mm.) is connected by means of a flexible rubber tube to a glass tube 20–25 cm. long. This glass tube reaches to the bottom of a receiver (of the type shown in the Figure) having a capacity of 250 c.c. The Kjeldahl flask has a mark indicating a volume of 30 c.c. and is lowered up to this mark into a conical air-bath. An asbestos plate with a circular hole protects the walls of the flask from overheating. The condenser tube and the connecting tube must be of pyrex glass. The diameter of the capillary tube of the dropping funnel is 0·8 mm. Thus, and attention must be paid to this, it is possible to produce regular dropping of the permanganate solution.

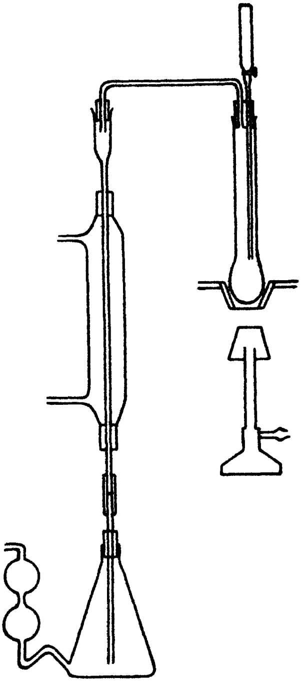

FIG. 33

Procedure.—Make the four determinations in succession. Pour

10 c.c. of the prepared solution (containing not more than 2 mg. of lactic acid) into the flask, neutralise with sulphuric acid and acidify with 1·5 c.c. of 5 per cent sulphuric acid. Then fill up to the 30 c.c. mark. In order to facilitate uniform boiling, add one or two pieces of porous pot of pepper corn size and a pinch of talc. Pour into the receiver 2 c.c. of 0·1 *N*-bisulphite solution diluted with 30 c.c. of doubly distilled water. Do not allow the extended condenser tube to dip into the bisulphite solution yet. Fill the stem of the dropping funnel with water and close the cock. Connect the flask with the condenser and heat with a Bunsen burner having a chimney. Keep the flame the same size throughout the whole course of the experiment. While heating, fill the dropping funnel with 0·002 *N*-permanganate solution. As soon as all the air has been expelled from the flask and from the upper part of the condenser tube (it is easy to note when this is so) attach the receiver to the condenser tube by means of a rubber stopper making the connexion air-tight, the tip of the condenser tube now dipping into the bisulphite solution. Now allow the permanganate solution to run in, drop by drop, at the rate of 0·8 c.c. per minute. Make sure that the liquid evaporates at the same rate so that the volume of liquid in the flask remains always 30 c.c. In order that further oxidation of the acetaldehyde may be avoided, never allow the liquid to become pink. Normally, the determination lasts altogether about 30 minutes.

At the end of the oxidation, the liquid becomes brown. When this is so, heat for 8–10 minutes longer continuing the addition of permanganate; disconnect the receiver so that the tube dips no longer into the bisulphite solution, and, without turning down the flame, boil for 1 minute longer in order to wash out the condenser tube with the water which distils. Then stop the heating, wash the extension tube with a few drops of distilled water, and, after 5–10 minutes, titrate the solution.

For this purpose add 1 c.c. of the starch solution, destroy most of the bisulphite with approximately 0·05 *N*-iodine solution and then titrate with the 0·01 *N*-iodine solution until the blue colour is just perceptible. If too much iodine solution is added, titrate back with a dilute solution of the bisulphite compound, added drop by drop. Now decompose the bisulphite compound by adding 0·5–1·0 g. of analytically pure sodium bicarbonate so that the solution becomes alkaline to litmus. Then repeat the titration until the blue colour appears. The calculation is based on the number of cubic centimetres of 0·01 N-iodine solution now used.

1 cubic centimetre of 0·01 N-iodine solution corresponds to 0·45 mg. of lactic acid. Determine the lactic acid content of the four samples, taking account of the increases in volume produced by removal of protein and carbohydrate, and by using aliquot portions at the various stages.

It will be found that the lactic acid content (" resting " lactic acid content) in 1 is considerably lower than in 2, 3 and 4, in which the contents are all of the same order of magnitude (about 20 mg.).

BACTERIAL LACTIC ACID PRODUCTION

Obtaining the Bacteria.—*B. Delbruckii* or *B. acidophilus* is used. Cultures of these bacteria for inoculation can be obtained from the National Collection of Type Cultures, London, from the Institut für Gärungsgewerbe in Berlin, or from dairy research institutes. The bacteria are propagated on sterile, perfectly clear, dark beer wort (*d* 1·0322) at a temperature of 40°. Since they are (facultative) anaerobic bacteria, the conical flasks (capacity 600 c.c.) used should be filled with the wort nearly to the level of the cotton-wool plugs, and, after the sterilisation, a few drops of the culture for inoculation, suspended in physiological salt solution, are run in from a sterile pipette. A copious growth appears within 1–2 days. This is removed from the wort by centrifuging in a high-speed centrifuge and is then stirred up twice with physiological salt solution, washed in the centrifuge and again suspended in the salt solution. The suspension thus obtained can easily be measured out as required, with a pipette. The experiments must be made at once with freshly harvested bacteria because these deteriorate on keeping. It is desirable also to test each culture microscopically by staining with carbofuchsine or gentian violet and comparing with the material obtained for inoculation.

Experiment.—The fermentation of glucose by lactic acid bacteria, with and without the addition of iodo-acetic acid, should be followed; the carbon dioxide liberated from calcium carbonate by the lactic acid produced being measured manometrically in a Warburg apparatus (see the experiment on p. 250).

The flasks are filled with a mixture of twenty parts by volume of carbon dioxide and eighty parts by volume of nitrogen. As was pointed out on p. 255, iodo-acetic acid inhibits glycolysis. Bacterial lactic acid fermentation is not excepted from this inhibition (Bertho[1]). When glycolysis is inhibited by iodo-acetic acid, respiration remains unaffected only at certain concentrations of the acid and when these are high, respiration also is considerably inhibited. In the present case $0{\cdot}002M$-iodo-acetic acid suffices completely to inhibit lactic acid production but this concentration also causes considerable inhibition of respiration. The inhibition of glycolysis reaches its maximum only after some time.

Procedure.—Prepare six samples each of 10 c.c. and each containing about 10 mg. of bacteria (dry weight) in the form of a suspension (volume, 1 or 2 c.c.) together with 1 c.c. of $0{\cdot}2M$-glucose and 0·2 g. of calcium carbonate. To each of three of the vessels, add also 1 c.c. of 0·02 M-solution of sodium iodo-acetate. Make up to a volume of 10 c.c. in all six vessels with physiological salt solution and pass the mixture of gases through each vessel for 5 minutes. Then shake for 15 minutes without taking readings, keeping the temperature at about 37°. Take readings thereafter every 15 minutes, convert the readings into cubic centimetres of CO_2 with the help of the constants K_{CO_2} and so determine the amount of lactic acid produced per hour and per gramme of bacteria. Note that 1 c.c. of CO_2 from the calcium carbonate corresponds to 8·0 mg. of lactic acid.

It is not only the true lactic acid bacteria, namely, those which convert sugar exclusively or almost completely into lactic acid, that produce this acid. Very many other bacteria from all groups of hydrolytic fungi are able to produce lactic acid, *together with other products*. It may be assumed that the mechanism of bacterial lactic acid production is fundamentally the same as or else very similar to that of lactic acid production in muscle (phosphorylation, dephosphorylation and so on). If Neuberg's assumption holds that here also methylglyoxal is the central substance in the process, then, in this case also, the conversion of the methylglyoxal into lactic acid must be brought about by keto-aldehyde mutase (see p. 257). The Pasteur-Meyerhof effect can also be observed in the case of lactic acid-producing bacteria with the important difference that the resynthesis leads to sugar not to glycogen (Meyerhof). With those bacteria which do not produce exclusively lactic acid but, like yeast, also produce C_2- and C_1-compounds (*B. coli*, for example) there exists, therefore, a fermentation mechanism in which the two main types of glycolysis (animal glycolysis type and yeast fermentation type) may be combined with each other in varying degrees.

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[1] *Annalen*, 1932, 494, 185.

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